A community analysis of dental plaque in pre-pubertal children

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Abstract

Healthy children, aged between 5 and 9 years who had not taken antibiotics in the preceding three months were recruited. Plaque was sampled from the gingival crevice of either the lower left or lower right first permanent molar to estimate the prevalence of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Tannerella forsythensis* which have been implicated as main etiological agents of periodontal disease. Analysis of plaque from subjects without gingivitis (n = 65) and those with gingivitis (n = 53) by PCR targeting of the 16S rRNA gene demonstrated negligible differences in the prevalence of these pathogens with the exception of *T. forsythensis*. This pathogen was detected more frequently in children with no gingivitis (P = 0.03). Community analysis of plaque was carried out to determine (i) any significant differences in the microbiota of both cohorts and (ii) if specific taxa influenced the prevalence of the three periodontal pathogens. Community analysis was attempted by using both culture dependent and culture independent techniques. The culture dependent technique involved community level physiological profiling (CLPP) and attempted to measure metabolic differences between cohorts. Preliminary studies demonstrated that this technique was not suitable for small sample volumes, such as plaque from single sites and was abandoned. The culture independent technique, denaturing gradient gel electrophoresis (DGGE), is a rapid and cost effective method for analysing bacterial communities. Statistical analysis of the DGGE data for both cohorts suggested that bacterial diversity was lower in subjects with gingivitis (P = 0.009). Logistic regression analysis of the DGGE banding patterns demonstrated that specific bands were significantly associated with gingival health and with gingivitis. Similarly, other bands were significantly associated with the prevalence of the three periodontal pathogens. These bands were excised and PCR-cloned. The 16S rRNA sequence data for these clones demonstrated that these DGGE bands were mixed with DNA of multiple taxa. Further attempts to separate individual excised bands over shorter denaturing gradients proved unsuccessful.

This work has demonstrated that *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* are present in the dental plaque of pre-pubertal children both with and without gingivitis. It has also provided valuable insights into the applications and limitations of fingerprinting techniques such as CLPP and DGGE.

Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigation, except where otherwise stated.

"Sometimes a scream is better than a thesis."

- Ralph Waldo Emerson (1803-1882)

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Thank you

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List of Abbreviations

16S rRNA gene	16S ribosomal Ribonucleic Acid gene
A.a	*Actinobacillus actinomycetemcomitans*
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Total
Bp	Base Pair
Bp-PEG	Bisbenzimide-Polyethyleneglycol
°C	Degrees Celsius
CLPP	Community Level Physiological Profiling
CDT	Cytolethal Distending Toxin
CTU	Co-Migrating Taxonomic Units
DAF	DNA Amplified Fingerprints
DGGE	Denaturing Gradient Gel Electrophoresis
DGGEGE	Denaturing Gradient Gel Electrophoresis Gel Expansion
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
FAB	Fastidious Anaerobic Broth
g	Grams
GAP	Generalised Aggressive Periodontitis
GCP	Generalised Chronic Periodontitis
GCF	Gingival Crevicular Fluid
GJP	Generalised Juvenile Periodontitis
h	Hour
H_2O	Water
H_2O_2	Hydrogen Peroxide
IL-1α	Interleukin 1 Alpha
IL-1β	Interleukin 1 Beta

IL-8	Interleukin 8
kb	Kilobase
KDa	Kilodaltons
LAP	Localised Aggressive Periodontitis
LCP	Localised Chronic Periodontitis
LJP	Localised Juvenile Periodontitis
LMW	Low Molecular Weight
LPS	Lipopolysacharide
µl	Microlitre
M	Molar
MCP-1	Monocyte Chemoattractant Protein 1
MAP	Mitogen Actiated Protein
min	Minute
ml	Millilitre
mM	Mili Molar
MMP	Matrix Metalloproteinase
NAM	N-Acetylmuramic Acid
NCTC	National Collection Of Type Cultures
NUG	Necrotising Ulcerative Gingivitis
NUP	Necrotising Ulcerative Periodontitis
OD	Optical Density
OTU	Operational Taxonomic Units
O_2	Oxyen
PCR	Polymerase Chain Reaction
Pg	*Porphyromonas gingivalis*
PHLS	Public Health Laboratory Services
PMN	Polymorphonuclear Leukocytes
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RTF	Reduced Transport Fluid
RTX	Repeat In Toxin

SSCP	Single Strand Conformation Polymorphism
T_A	Annealing Temperature
T_M	Melting Temperature
TAE	Tris Acetate EDTA
Tf	*Tannerella forsythensis*
TGGE	Temperature Gradient Gel Electrophoresis
TH	Todd Hewitt
TIMPS	Tissue Inhibitors Of Metalloproteinases
tRFLP	Terminal Restriction Fragment Length Polymorphism
UV	Ultra Violet
v/v	Volume for Volume

Chapter 1: Introduction

1.1 Microbial colonisation of the oral cavity

1.1.1 Development of the microbiota

The oral cavity of neonates tends to be sterile at birth, despite exposure to the maternal resident flora of the cervix, uterus, vagina and perineum (Carlsson and Gothefors, 1975). The newborn becomes exposed to micro-organisms from a variety of sources such as water, food, breast milk and in particular saliva transferred to the infant from the mother (Long and Swenson, 1976; Kononen et al., 1992; Wan et al., 2001). Most of these bacteria are transient with only a small proportion becoming established as the normal flora. Primary colonisation of the oral cavity in predentate children is dominated by the streptococci, in particular *Streptococcus mitis* biovar 1 and *Streptococcus salivarius* (Smith et al., 1993). The mucosal surfaces of the oral cavity are the only available surfaces for colonisation in the first few months of life. With eruption of the primary teeth, these hard (non-shedding) surfaces present more suitable niches for bacterial colonisation. Furthermore, the eruption of teeth introduces gingival crevicular fluid (GCF), a tissue exudate that bathes the periodontal tissue, as an additional source of nutrients.

The major surfaces present in the oral cavity on which ecosystems can form include the buccal epithelium, dorsum of the tongue, hard and soft palate, tonsils, pharynx, supragingival tooth surface (above the gum margin) and subgingival tooth (below the gum margin) and crevicular epithelial surfaces (figure 1.1). The richness and proportions of the oral microbiota differ between each site in the oral cavity (Mager et al., 2003). This is because the oral cavity provides a series of varied ecosystems as a result of differences in the physical and chemical features present. Examples of varying parameters include temperature, oxygen tension, pH, surface chemistry and nutrient availability (Schonfeld, 1992). Within these environments, microenvironments exist e.g. supragingival plaque can be subdivided into gingival, approximal and fissure plaque (figure 1.2), each of which is optimal for colonisation and growth of different micro-organisms (Marsh and Martin, 1999).

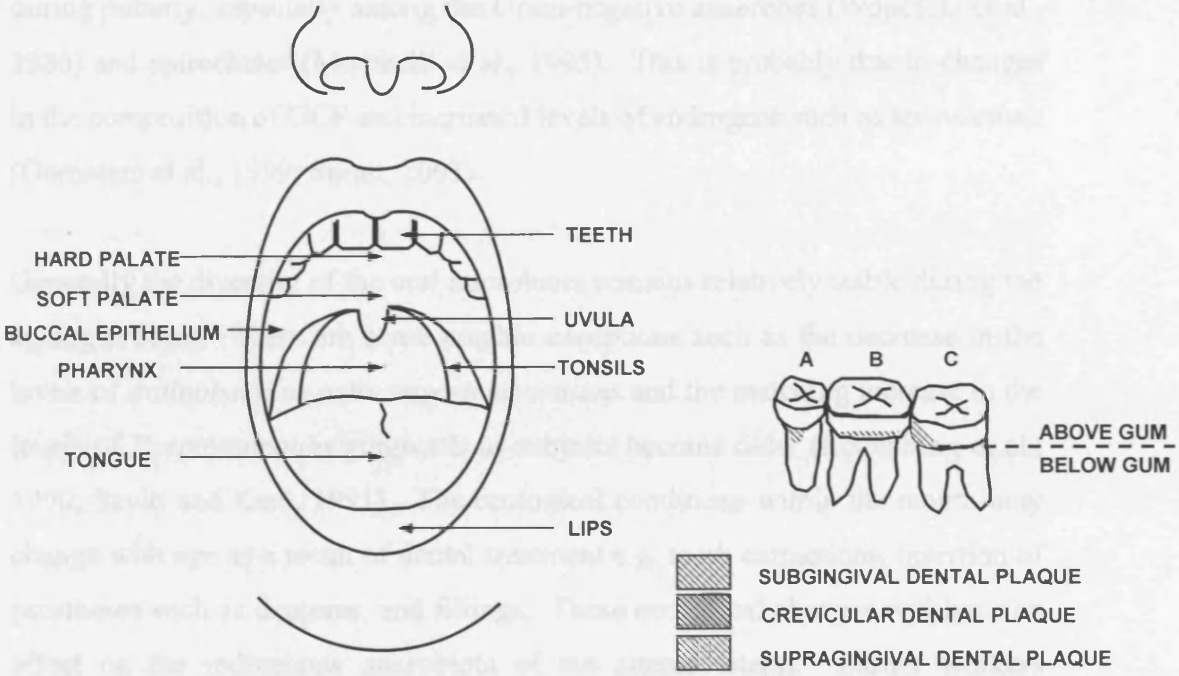


Figure 1.1: Different oral surfaces for microbial colonisation: (A) Subgingival plaque, (B) Crevicular plaque and (C) Supragingival plaque

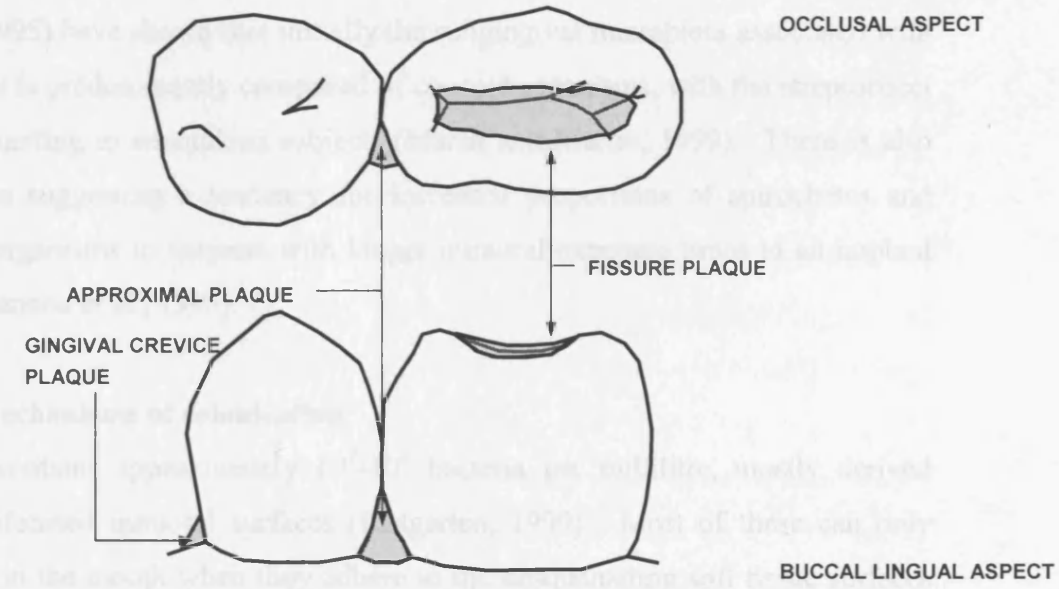


Figure 1.2: Areas of dental plaque accumulation: Different microenvironments composing supragingival dental plaque (adapted from Marsh and Martin, 1999)

The diversity of the oral microbiota of dentate infants (6-18 months) increases with time (Kononen et al., 1994, 1999; Spratt, 2003). This is particularly so during puberty, especially among the Gram-negative anaerobes (Wojicicki et al., 1986) and spirochetes (Mombelli et al., 1995). This is probably due to changes in the composition of GCF and increased levels of androgens such as testosterone (Gornstein et al., 1999; Spratt, 2003).

Generally the diversity of the oral microbiota remains relatively stable during the ageing process. There are some notable exceptions such as the decrease in the levels of *Actinobacillus actinomycetemcomitans* and the matching increase in the levels of *Porphyromonas gingivalis* as subjects become older (Rodenburg et al., 1990; Savitt and Kent, 1991). The ecological conditions within the mouth may change with age as a result of dental treatment e.g. tooth extractions, insertion of prostheses such as dentures, and fillings. These ecological changes will have an effect on the indigenous microbiota of the altered site(s). Earlier workers (Nogueira et al., 2000) have demonstrated that supragingival plaque control in patients with gum disease results in a significant increase in coccoid cells and a significant decrease in motile rods and *spirochetes*. Other workers (Papaioannou et al., 1995) have shown that initially the subgingival microbiota associated with implants is predominantly composed of coccoid organisms, with the streptococci predominating in edentulous subjects (Marsh and Martin, 1999). There is also evidence suggesting a tendency for increased proportions of spirochetes and motile organisms in subjects with longer intraoral exposure times to an implant (Papaioannou et al., 1995).

1.1.2 Mechanisms of colonisation

Saliva contains approximately 10^7-10^8 bacteria per millilitre, mostly derived from colonised intraoral surfaces (Listgarten, 1999). Most of these can only survive in the mouth when they adhere to the desquamating soft tissue surfaces or the non-desquamating hard surfaces (teeth, implants and dentures). Oral bacteria are dependent on adhering mechanisms to successfully colonise the oral cavity. In their absence, bacteria would join the salivary flow and be swallowed. There is a dynamic equilibrium between the adhesive forces of microbes onto these intraoral surfaces and a variety of removal forces: (i) mechanical forces

such as swallowing and mastication, (ii) the use of tongue and oral hygiene implements such as toothbrushes and (iii) the wash-out effect of the salivary, nasal and crevicular fluid outflow (Quirynen et al., 2001). As a consequence of this dichotomy, the incumbent microbiota of differing intraoral surfaces are different (Marsh, 1994; Ximenez-Fyvie et al., 2000b; Mager et al., 2003).

The first substances to become associated with the colonisable surface are not bacteria but an array of chemical complexes found in the ambient fluid milieu that covers the tooth and epithelial cell surfaces (Whittaker et al., 1996). This leads to the formation of a host-derived layer known as the acquired pellicle (or conditioning film) which starts forming on the enamel and other exposed tissues of teeth immediately after tooth brushing (Schupbach et al., 2001). The pellicle is an acellular organic film less than 1 μm in thickness and is formed from numerous components in saliva, which include glycoproteins, fibronectin, mucins, proline-rich proteins, histidine-rich proteins, a number of enzymes such as α-amylase, phosphate-containing proteins like statherin, as well as other molecules (Whittaker et al., 1996; Listgarten, 1999). Plaque development on the acquired pellicle can be divided into four main stages (Marsh, 1991). The first stage of plaque development is reversible and involves the attraction of bacterial cells to the substratum (attaching surface). The acquired pellicle alters the free energy and charge of the substratum and thus attracts bacteria suspended in the saliva via van der Waals forces as well as other electrostatic interactions. In this phase of loose association the bacteria are located approximately 10 nm from the substratum surface (Gibbons, 1984). These ionic bonds, as well as other physical forces of low specificity, may not be sufficient for colonisation (Gibbons, 1984).

The second stage of plaque development is irreversible and involves adhesion of the microorganism to the acquired pellicle. Adhesion is the process by which bacteria "selectively" colonise specific surfaces (Gibbons, 1984). For example, *Streptococcus oralis*, *Streptococcus mitis* and *Streptococcus constellatus* have been reported to preferentially colonise soft, desquamating tissues and can also be found in saliva in higher proportions than on tooth surfaces (Mager et al., 2003). Conversely *Streptococcus gordonii*, *Selenomonas noxia*, *Streptococcus sanguinis* and *Streptococcus anginosus* have been detected in higher numbers

adhering to tooth surfaces (Mager et al., 2003). These studies strengthen the notion that bacteria possess highly developed recognition systems on their cell surface which enable them to identify and interact with different components of the different intraoral surfaces. Principal early or primary colonisers include streptococci and *Actinomyces* spp (Nyvad and Kilian, 1987; Palmer, Jr. et al., 2003). These bacteria can synthesise specific ligands known as adhesins that are often present on their cell surfaces and which enable binding to the acquired pellicle (Gibbons, 1984). Adhesion occurs via lectin-like and/or hydrophobic interactions between adhesins on the bacterial cell surface and receptors on the acquired pellicle. Frequently these are presented in filamentous surface appendages such as pili or fimbriae (Jin and Yip, 2002). *Actinomyces* spp (Hallberg et al., 1998; Li et al., 1999) as well as streptococcal species (Gibbons et al., 1991; Jenkinson and Lamont, 1997) have been demonstrated to bind to acidic proline-rich proteins and statherin receptors. Multiple salivary components, including the low molecular-weight salivary mucin and α-amylase, promote the adhesion of organisms such as the streptococci (Murray et al., 1992). The glycoprotein fibronectin can also promote the adhesion of oral bacteria such as *Fusobacterium nucleatum* and streptococcal species (Winkler et al., 1987). These first two stages of plaque development are summarised in figure 1.3.

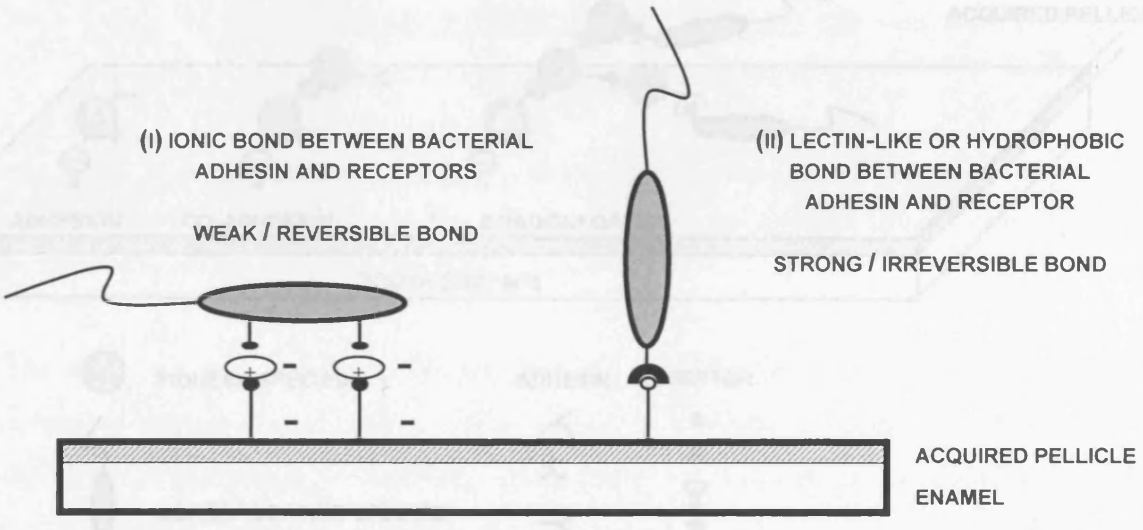


Figure 1.3: Mechanisms of bacterial attraction and adhesion onto the acquired pellicle

As well as binding to desquamating and non-desquamating surfaces of the oral cavity, bacteria can also adhere to the nascent bacterial layers that initially coat the teeth and epithelial cells. This adherence of microorganisms to already attached cells is the third stage of plaque development. In suspension, adherence between different bacteria is termed as co-aggregation. These specific partnerships between dental plaque bacteria are very common, especially between the primary colonisers that can coaggregate with each other but not usually with secondary colonisers (Rickard et al., 2003). When one partner of the adhering bacterial partnership is bound to the pellicle then this type of association is referred to as co-adhesion (Jin and Yip, 2002). Co-adhesion appears to be of great importance for the microbial colonisation of tooth surfaces. For example, earlier workers (Ganeshkumar et al., 1991) have demonstrated that *S. sanguinis* strains facilitate the adherence of *Actinomyces naeslundii*. As each new cell type adheres its cell body becomes a nascent surface which in turn presents numerous possible receptors and adhesins for other bacterial strains. Coaggregation may occur between bacteria of the same genus (intrageneric) and/or between different genera (intergeneric) (figure 1.4).

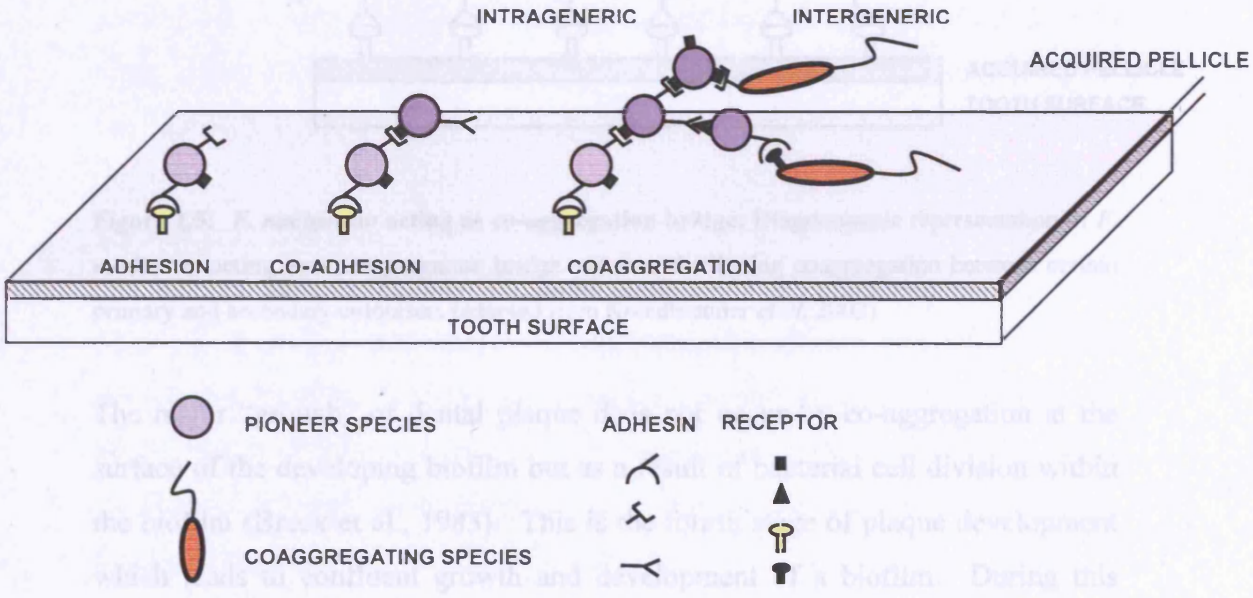


Figure 1.4: Bacterial adhesion, co-adhesion and co-aggregation

Other workers have postulated that anaerobic bacteria, in dental plaque, can actively co-exist in aerobic environments as a result of co-aggregation with oxygen-utilising aerobes and facultative species within the biofilm (Bradshaw et al., 1998). The juxtapositioning between diverse microorganisms is believed to be facilitated by organisms such as *F. nucleatum*. These act as 'co-aggregation bridges' (figure 1.5) between non-coaggregating primary and secondary colonisers (Bradshaw et al., 1998) for a large subset of oral bacteria (Kolenbrander et al., 1989, 2002). In the absence of bacteria such as *F. nucleatum* that act as co-aggregation bridges, many secondary colonisers might not be capable of becoming part of the dental plaque community.

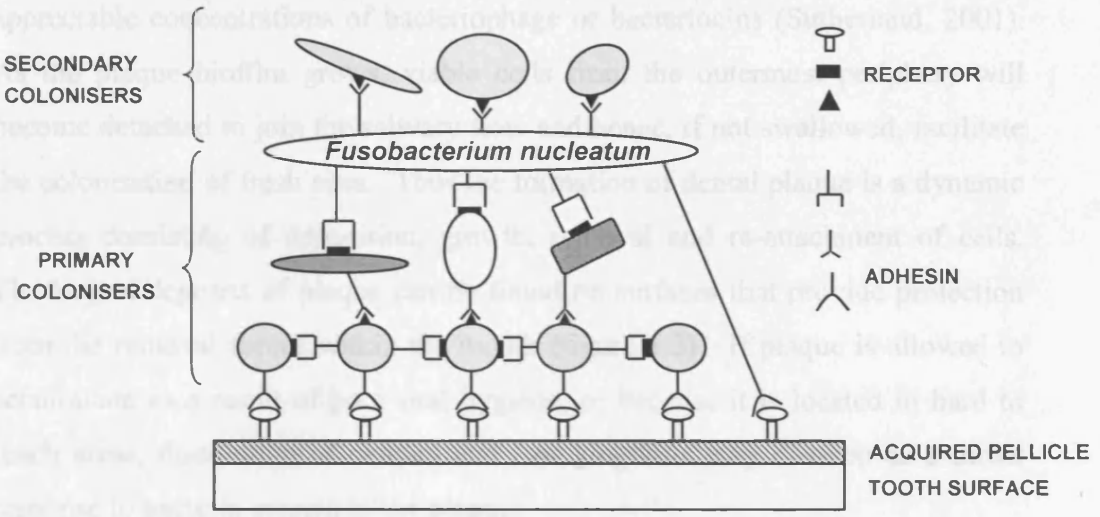


Figure 1.5: *F. nucleatum* acting as co-aggregation bridge: Diagrammatic representation of *F. nucleatum* acting as a coaggregation bridge and thus facilitating coaggregation between certain primary and secondary colonisers (adapted from Kolenbrander *et al*, 2002)

The major "growth" of dental plaque does not occur by co-aggregation at the surface of the developing biofilm but as a result of bacterial cell division within the biofilm (Brecx et al., 1983). This is the fourth stage of plaque development which leads to confluent growth and development of a biofilm. During this growth stage, the bacterial community will shift from predominantly cocci to a more complex system with increased numbers of rods, filamentous organisms and spirochetes (Marsh, 1991).

An important physiological process that usually occurs during the development of dental plaque biofilms and is believed to be important for the adhesion of secondary colonisers is the increased production of extracellular polymeric substances (EPS). These polymers not only envelop the attached cells within the biofilm but also strengthen adhesion between cells in the biofilm and may also act as receptors for co-aggregation interactions (Rickard et al., 2003). Further to this, EPS from oral biofilms may convey some degree of protection to the 'encased' bacterial cells within. Some workers have suggested that EPS in biofilms may interact with antimicrobial agents and thus protect the cells, either by preventing access of the compounds or by effectively reducing the concentration (Sutherland, 2001). EPS may offer little protection against appreciable concentrations of bacteriophage or bacteriocins (Sutherland, 2001). As the plaque biofilm grows, viable cells from the outermost periphery will become detached to join the salivary flow and hence, if not swallowed, facilitate the colonisation of fresh sites. Thus the formation of dental plaque is a dynamic process consisting of deposition, growth, removal and re-attachment of cells. The largest deposits of plaque can be found on surfaces that provide protection from the removal forces within the mouth (figure 1.2). If plaque is allowed to accumulate as a result of poor oral hygiene, or because it is located in hard to reach areas, dissolution of enamel and / or gingivitis may develop as a direct response to bacteria present in the plaque.

1.2 The periodontium

The healthy periodontium provides the support required for appropriate tooth function. It is comprised of four principal structures (figure 1.6), which are the periodontal ligament, alveolar bone, cementum and gingivae (Bartold et al., 2000). Each of these components are distinct with respect to location, tissue architecture as well as biochemical and cellular composition, and yet they function as a single unit (Bartold et al., 2000).

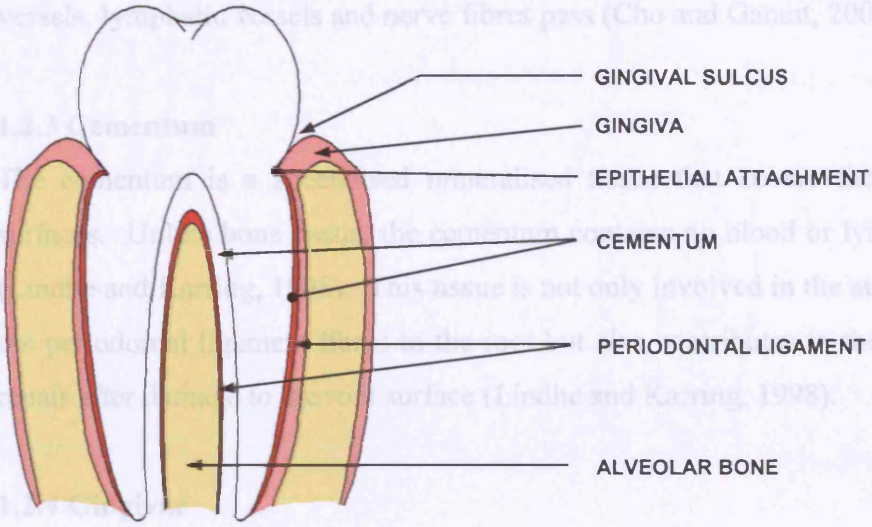


Figure 1.6: Diagrammatic representation of the periodontium and surrounding structures

1.2.1 Periodontal ligament

The periodontal ligament is the soft, richly vascular and cellular connective tissue that surrounds the roots of the teeth and joins the root cementum with the alveolar bone. This ligament is physically small but functionally important for both tooth support as well as the regulation of alveolar bone volume (McCulloch et al., 2000). An important feature of this connective tissue is its ability to adapt to alterations in mechanical loading. This feature is mediated in part by a heterogeneous cell population that enables the root of the teeth to maintain strong attachments to bone in spite of highly variable applied forces (McCulloch et al., 2000). The mature periodontal ligament can be subdivided into 3 main regions: (i) a bone-related region which is rich in cells and blood vessels, (ii) a cementum-related region which is comprised of dense, well-ordered collagen bundles and (iii) a middle zone containing less cells as well as thinner collagen fibrils (Cho and Garant, 2000).

1.2.2 Alveolar bone

The alveolar bone houses the roots of teeth and absorbs and distributes the pressure generated during tooth contacts (Cho and Garant, 2000). The most important function of the alveolar bone is to anchor the roots of teeth. The

coronal and apical regions of the alveolar bone are sieve-like in structure and connect the periodontal ligament to the bone marrow through which blood vessels, lymphatic vessels and nerve fibres pass (Cho and Garant, 2000).

1.2.3 Cementum

The cementum is a specialised mineralised tissue that covers the tooth root surfaces. Unlike bone tissue, the cementum contains no blood or lymph vessels (Lindhe and Karring, 1998). This tissue is not only involved in the attachment of the periodontal ligament fibres to the root but also contributes to the process of repair after damage to the root surface (Lindhe and Karring, 1998).

1.2.4 Gingivae

The gingivae are comprised of gingival epithelium and connective tissue. The gingivae form part of the oral mucosa that covers the tooth bearing part of the alveolar bone and cervical neck of the tooth (Cho and Garant, 2000). In health, the gingivae normally cover the alveolar bone and tooth root at a level that is just coronal to the cemento-enamel junction (figure 1.7). Normally healthy gingivae (figure 1.8) are pink in colour, interdentally firm and often exhibit a stippled appearance with a knife-edged margin between the soft tissue and the tooth. They do not bleed on gentle probing and fill in the space below the contact areas between the teeth (Kinane, 2001). The anatomy of the gingivae can be divided into 3 distinct domains: (i) the free marginal gingivae, (ii) the interdental gingivae and (iii) the attached gingivae (figure 1.8).

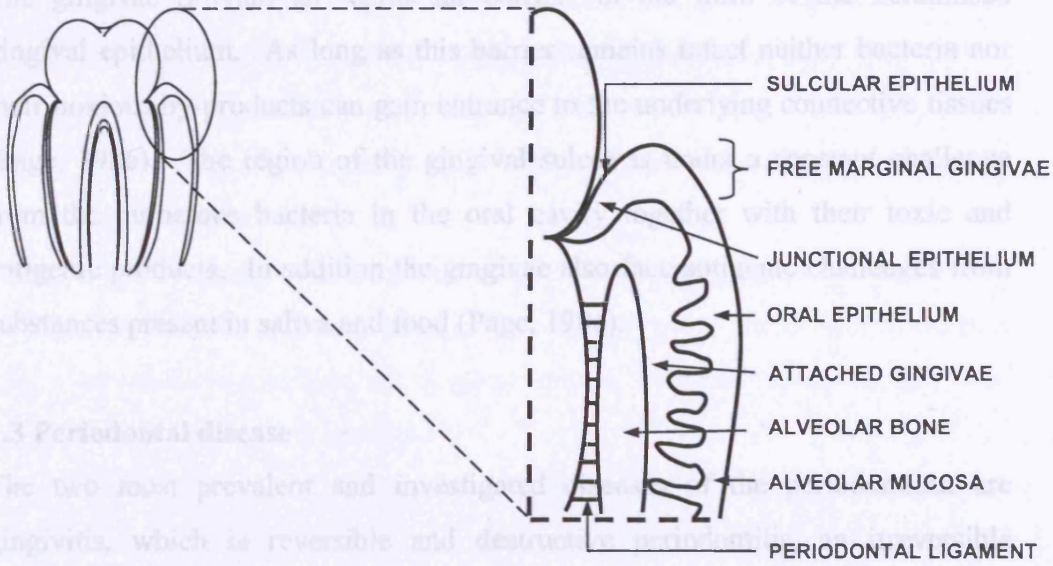


Figure 1.7: Anatomic relationship of gingival tissue to the teeth and alveolar bone

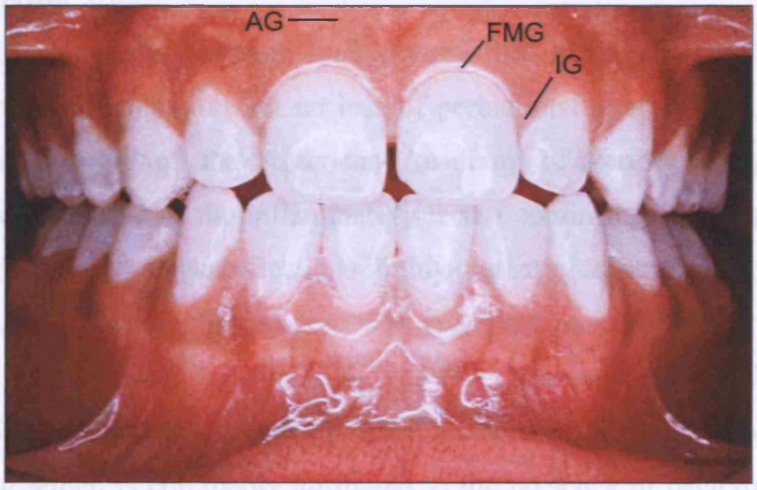


Figure 1.8: Healthy gingivae. Various clinical landmarks of the gingivae such as the free marginal gingivae (FMG), the interdental gingivae (IG) and the attached gingivae (AG) (photograph kindly provided by Professor Graham Roberts)

The gingivae provide an 'epithelial barrier' in the form of the keratinised gingival epithelium. As long as this barrier remains intact neither bacteria nor their noxious by-products can gain entrance to the underlying connective tissues (Page, 1986). The region of the gingival sulcus is under a constant challenge from the numerous bacteria in the oral cavity together with their toxic and antigenic products. In addition the gingivae also face antigenic challenges from substances present in saliva and food (Page, 1986).

1.3 Periodontal disease

The two most prevalent and investigated diseases of the periodontium are gingivitis, which is reversible and destructive periodontitis, an irreversible condition that can lead to tooth loss (Kinane, 2001). The unequivocal role of dental plaque in the development of these diseases has been well documented (Löe et al., 1965; Hoover and Lefkowitz, 1965; Beck et al, 1992; Greenstein and Lamster, 1997; Kamma et al, 2004).

1.3.1 Gingivitis

Traditionally, gingivitis has been limitedly ascribed to the clinical situation in which there is inflammation but no loss of periodontal attachment. Under the classification system of the American Academy of Periodontology (1986), gingivitis was defined as the inflammatory lesion confined to the tissues of the marginal gingivae. The classifications in this glossary had several shortcomings and thus a new classification system was presented in a 1999 workshop (The American Academy of Periodontology, 1999; Armitage, 1999; Wiebe and Putnins, 2000). Gingivitis is now observed to represent a spectrum of diseases, the onset of which is commonly attributed to the presence of bacteria in dental plaque (Löe and Holm, 1965; Socransky, 1970; Lindhe et al., 1973; Bosman and Powell, 1977; Page, 1986; Breuer and Cosgrove, 1989; Mariotti, 1999) but there are other forms of gingivitis that are not primarily plaque-related (Holmstrup, 1999). Non-plaque induced gingival lesions can result from specific bacterial, viral and fungal infections (Holmstrup, 1999). These non-plaque induced gingival lesions are much less prevalent. Gingival diseases associated with plaque, systemic diseases, endogenous hormonal fluctuations, drugs and

malnutrition have several essential characteristics in common that can be used as describing features of this condition in clinical assessments:

(i) Gingival diseases are confined to the gingivae

(ii) Dental plaque is present to either initiate or exacerbate the severity of the gingival lesion

(iii) Clinical signs for inflammation include enlarged gingival margins due to fibrosis, colour change from pink (healthy) to red and/or bluish-red hue, elevated temperature at gingival sulcus, bleeding when probing and increased gingival exudate

(iv) Stable attachment levels of the periodontium with no attachment loss

(v) Gingival disease is reversible on the removal of the aetiology/-(ies)

The extent of gingivitis may be defined as the total amount of inflamed gingival tissue within the mouth of a subject (Jackson, 1965). Visual assessment of gingivitis is limited to the extent and severity of changes in the physical status of the gingivae, such as changes in colour and swelling (Jackson, 1965) and in bleeding tendency (figure 1.9) (Kinane, 2001; Armitage, 2004b). Recognition of gingival swelling or oedema (figure 1.10) as well as gingival redness requires that the clinician have a clear mental image of the shape, texture and colouring of healthy gingivae (figure 1.8). These are qualitative rather than quantitative measures and the severity of gingivitis requires an arbitrary grading index. Several indices have been proposed for the clinical evaluation of gingival inflammation. These include the papilla, marginal, attached index (Massler, 1967), the papillary bleeding score (Loesche, 1979) and the papillary bleeding index (Muhlemann, 1977) amongst many others (Jackson, 1965; Tatakis and Trombelli, 2004). These methods all visually assess gingivitis with respect to histopathological changes of the tissue. Another clinical sign of gingivitis is an increased exudate of GCF (Löe and Holm, 1965). GCF typically flows at a rate of 3-6 $\mu l\ hr^{-1}$ per healthy tooth (Griffiths et al., 1992). Inflamed gingivae are observed to exude GCF at increased rates, the amount of which varies according to the severity of the inflammation (Löe and Holm, 1965; Del Fabbro et al., 2001; Weidlich et al., 2001). For such a reason the rate of GCF exudation and its quantitation (Ciantar et al., 2002) have been used to add further objectivity to the clinical assessment status of the gingivae in health and disease (Lamster, 1997;

Jin et al., 2003; Ozmeric, 2004; Armitage, 2004a). All these clinical signs of gingival inflammation must be associated with stable attachment levels on a periodontium when considering gingivitis. Moreover, no loss of attachment or of alveolar bone should be observed. Another universal feature of gingivitis is that unlike periodontitis the inflammation should be reversible upon the removal of the aetiological factors. This has been demonstrated in a study of experimental gingivitis which was reversed following removal of the main aetiological factor, dental plaque (Bosman and Powell, 1977).

Figure 1.9: Gingival bleeding. Spontaneous bleeding (GB – gingival bleeding) from the inflamed gingival margins of the upper incisor teeth (photograph kindly provided by Professor Graham Roberts)

Figure 1.10: Gingival redness and swelling. Heavy deposits of plaque and inflammation of the gingival margins of the upper incisor teeth. There is noticeable oedema of the interdental papillae (photograph kindly provided by Professor Graham Roberts)

1.3.2 Different types of dental plaque-induced gingival diseases

1.3.2.1 Induced by dental plaque

A) *Gingivitis attributed with only dental plaque*

(i) With no other local contributing factors – i.e. as a result of poor oral hygiene.

(ii) With local contributing factors – Supragingival irregularities such as crowding calculus or rough restorations (among many other factors) promotes the retention of dental plaque. These structures may also confer protection to the organisms within plaque from salivary enzymes and oral hygiene. The undisturbed bacteria may then illicit a host response resulting in the inflammation of the gingivae. Local factors are defined as any factor that influences periodontal health at a particular site or sites, with no systemic effects (Matthews and Tabesh, 2004).

1.3.2.2 Modified by systemic factors

(A) *Gingivitis attributed to the Endocrine system* – Physiological and pathological changes of the endocrine system have been reported as significant contributory factors in the expression of periodontal disease. Several examples include the following:

(i) Puberty-associated gingivitis – Past workers (Gornstein et al., 1999) have demonstrated that elevated levels of androgens such as testosterone and dihydrotestosterone may modulate the development of gingival inflammation.

(ii) Menstrual cycle-associated gingivitis – Fluctuation in oestrogen/progesterone levels have been shown to affect the periodontium. Previous workers (Machtei et al., 2004) have observed changes in gingival health during the menstrual cycles of periodontally healthy women.

(iii) Pregnancy-associated gingivitis – In a woman's life, the major physiological and hormonal changes occur in pregnancy. The most significant hormonal change is the increased production of oestrogen and progesterone. Periodontal infections have been reported to accelerate as a result of pregnancy (Laine, 2002; Lieff et al., 2004). Pregnancy gingivitis is an acute form of gingivitis that affects pregnant women (Barak et al., 2003). It is characterised by

erythema, oedema, hyperplasia and an increased bleeding of the gingival tissue. The prevalence is 30-75% of all pregnant women (de Liefde, 1984; Levin, 1987). Although gingivitis in pregnancy appears to be connected to pregnancy-related hormones, the exact mechanisms by which these hormones increase gingival inflammation remains unclear (Laine, 2002).

(iv) Diabetes mellitus-associated gingivitis – Diabetes mellitus is a systemic disease which presents the patient with several complications, one of which is periodontal disease (Iacopino, 2001; Nishimura et al., 2003; Arrieta-Blanco et al., 2003). Diabetes-associated gingivitis can be described as an inflammatory response of the gingivae to plaque aggravated by poorly controlled plasma glucose levels.

(B) *Gingivitis attributed to genetic abnormalities* – Genetic conditions can predispose subjects to an increased susceptibility to plaque-induced gingival diseases. Several examples include the following:

(i) Blood dyscrasias – Gingivitis may be an oral manifestation of leukaemia (Wu et al., 2002). Severe gingivitis is also a major feature among all patients suffering from leukocyte adhesion deficiency (LAD), a condition in which neutrophils are unable to aggregate and bind to adhesion molecules on endothelial cells (Holland and Gallin, 1998; Lakshman and Finn, 2001).

(ii) Down's syndrome-associated gingivitis – Previous workers (Lopez-Perez et al., 2002) have demonstrated that the extent and severity of gingivitis was greater in Down's syndrome subjects as opposed to non-Down's syndrome control subjects.

1.3.2.3 Modified by environmental factors

(A) *Smoking* – This is an environmental factor that has been well documented to influence the expression of plaque-related gingivitis. In experimental gingivitis studies both smokers and non-smokers accumulate plaque at the same rate but smokers exhibit less gingival inflammation (Giannopoulou et al., 2003). Furthermore, other workers (Nair et al., 2003) have demonstrated that gingival bleeding on provocation increases after patients quit smoking. Smoking has been

demonstrated to interfere with cytokine production (Giannopoulou et al., 2003) and therefore it is possible that gingivitis may be less common in smokers as a result of a dampened host response to dental plaque. Furthermore, other workers (Villar and de Lima, 2003) have proposed that this reduction of inflammation in smokers may be associated with local vasoconstriction and an increased gingival epithelial thickening as a consequence of smoking.

(B) *Drug consumption* – Various medications have been demonstrated to have an adverse effect on the gingivae. Certain drugs such as the anti-convulsant phenytoin, the immunosuppressant cyclosporin and calcium channel blockers such as nifedipine have been implicated in gingival overgrowth (Seymour et al., 1996). Other drugs have been shown to curtail plaque-induced gingival inflammation. The non-steroidal anti-inflammatory drug (NSAID) flurbiprofen can act by inhibiting the host inflammatory response, thus inhibiting the development of gingivitis. Drugs such as cetylpyridinium chloride can inhibit plaque from forming and thus prevents the ensuing gingivitis (Tatakis and Trombelli, 2004).

1.3.2.4 Modified by malnutrition

It is believed that nutritional deficiencies might exacerbate the response of the gingivae to bacteria in dental plaque (Mariotti, 1999). Ascorbate (vitamin C) deficiency has been previously demonstrated to influence the early stages of gingival inflammation (Jacob et al., 1987).

1.3.3 Necrotising ulcerative gingivitis

Necrotising ulcerative gingivitis (NUG) is a type of necrotising periodontal disease in which the necrosis is limited to the gingival tissue (figure 1.11). The clinical characteristics that are used to diagnose for NUG include (i) pain, (ii) inter-dental necrosis and (iii) bleeding (Rowland, 1999).

Previous researchers have demonstrated several predisposing factors that encourage NUG, these include psychological stress (Moulton et al., 1952), immunosuppression (Cogen et al., 1983) and malnutrition (Johnson and Engel, 1986).

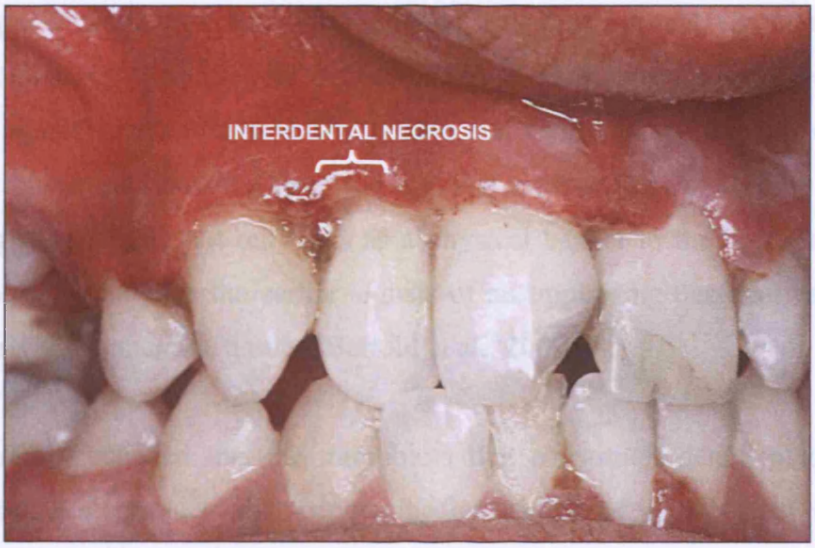


Figure 1.11: Necrotising ulcerative gingivitis (NUG). Clinical photograph of patient with NUG. The subject presents inter-dental necrosis with no loss of clinical attachment or alveolar bone (photograph kindly provided by Professor Stephen Porter)

1.4 Host-microbe interactions in gingivitis

1.4.1 The role of the host response in gingivitis

Experimental gingivitis studies (Bosman and Powell, 1977) have demonstrated that plaque accumulation will illicit gingival inflammation that can be reversed by re-establishing adequate oral hygiene. The gingivae provide the first line of defence against the bacteria within plaque and increased production of GCF helps to "wash out" bacteria from the gingival crevice (Löe and Holm, 1965). The gingival epithelia not only acts as a physical barrier to the bacteria within plaque but also initiates the earlier signals of an impending bacterial assault to the underlying connective tissues (Bartold et al., 2000).

It is currently believed that the microbiota that constitutes dental plaque will release bacterial products that trigger the expression of a variety of immune response mediators from epithelial cells (Okada and Murakami, 1998). These mediators play a major role in acute or chronic inflammation and are involved in the initiation, perpetuation and eventually the termination of an inflammatory response (Kinane et al., 2001). Examples of these bacterial products include short chain fatty acids, which are major by products of microbial metabolism (Gorbach et al., 1976). Previous workers (Kurita-Ochiai et al., 1995) have demonstrated that volatile fatty acids such as butyric, propionic, valeric and isovaleric acids, produced by oral pathogens, greatly inhibit murine T- and B-cell proliferation as well as significantly depressing the production of cytokines. Further work has demonstrated that these fatty acids also induce apoptosis of T- and B-cells (Kurita-Ochiai et al., 1997, 1998). These metabolic by-products are considered to be important virulence factors as they play a significant role in depressing the host immune response against periodontal pathogens.

Other bacterial products include a large variety of soluble enzymes that can digest host proteins and other molecules to produce nutrients for growth (Kinane et al., 2001). One such enzyme group includes the proteases which can digest a wide array of host tissues. Proteases are proteolytic enzymes that may be divided into 2 major classes: (i) endopeptidases and (ii) exopeptidases, depending on the location of activity of the enzyme on its substrate (Kinane and Lindhe, 1998). Endopeptidases cleave their substrates within the polypeptide chains, whilst the

exopeptidases cleave their substrates at the end of the polypeptide chain (Kinane and Lindhe, 1998). An example of these proteases includes the gingipains (Potempa et al., 2003). These gingipains, which have been widely documented in the periodontal pathogen *P. gingivalis* (Potempa et al., 1995; Travis et al., 1997; Genco et al., 1999; Potempa et al., 2003), are present on the cell surface of this putative pathogen. Gingipains belong to the family of extracellular cysteine proteases, which are products of three genes, two coding for an Arg-specific (RgpA and RgpB) and one for Lys-specific proteases (Kgp) (Potempa et al., 2003). The Arg- and Lys-gingipain cysteine proteases are the main endopeptidases produced by *P. gingivalis* (Sojar et al., 1999). Previous workers (Genco et al., 1999) have stressed the potential roles of Arg- and Lys-gingipains of *P. gingivalis* in periodontal disease in that they appear to be associated with host tissue destruction. In addition to providing amino acids, peptides and haemin for growth, protease activity is also believed to provide substrates for cell surface adhesion (Kadowaki et al., 1994; Lamont and Jenkinson, 2000).

As well as secreting their own proteases, oral bacteria present in dental plaque can also stimulate the expression of proteases from host tissue cells. An example of such proteases include the matrix metalloproteinases (MMPs), which are a family of calcium-dependant, zinc-containing endopeptidases that are structurally and functionally related (Bode and Maskos, 2003). The activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) are the most important (Brew et al., 2000). MMPs play an important role in the normal physiology of connective tissue during development, morphogenesis and wound healing (Heikinheimo and Salo, 1995; Chin and Werb, 1997; Pilcher et al., 1999; Steffensen et al., 2001). Their unregulated activity has been implicated in numerous disease processes including arthritis, tumour cell metastasis and atherosclerosis (Brew et al., 2000). Different workers (Uitto et al., 1998) have demonstrated that in periodontitis, collagenase 3, a potent MMP, is expressed in the gingival pocket by epithelial cells. Specific periodontal pathogens such as *Fusobacterium nucleatum* have been demonstrated to induce the expression of collagenase 3 in epithelial cells (Uitto et al., 2005). Other MMPs, such as MMP-2 have also been implicated in the pathogenesis of periodontal disease (Korostoff et al., 2000; Tiranathanagul et al., 2004). It has

been demonstrated that the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans* can activate MMP-2 in human periodontal ligament cells (Tiranathanagul et al., 2004). The family of human MMPs consist of 23 different forms that are divided into 6 groups (Snoek-van Beurden and Von den Hoff, 2005). These diverse MMPs target different substrates, which include collagen, gelatin, fibronectin, laminin, elastin, aggrecan, and casein (Snoek-van Beurden and Von den Hoff, 2005).

The hosts defence mechanism against infectious agents can be divided into 2 broad divisions, the innate (non-specific) and the adaptive (specific) responses. Innate reactions include the inflammatory response and do not involve immune mechanisms. Adaptive responses involve specific immune responses to pathogens (Kinane et al., 2001). The persistence of infection in spite of the action of the innate immune response leads to the induction of an adaptive immune response. The adaptive immune response can be subdivided into humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies, whereas cell-mediated immunity involves the direct action of immune cells (Kinane et al., 2001). Supragingival and subgingival plaque, as well as the cell surface components from Gram-negative and Gram-positive bacteria have been demonstrated to invoke both the inflammatory and immune responses as they interact with host cells (Taichman et al., 1977; Nassar et al., 2002).

Host polymorphonuclear leukocytes (PMNs) have been demonstrated to release lysosomal constituents upon *in vitro* exposure to either viable or irradiated, supragingival or subgingival dental plaque (Taichman et al., 1977). Electron microscopic observations from this study revealed that PMNs might be associated with phagocytosis of both Gram-negative and Gram-positive bacteria from dental plaque. Moreover, it is also believed that a specific microbiota of both supragingival and subgingival plaque might stimulate a dose-dependant release of oxygen metabolites (O_2-, H_2O_2, HOCl, etc) from isolated human PMNs (Taichman et al., 1977; Seow et al., 1992). Lysosomes from PMNs contain proteases, carbohydrases, hydrolases and antimicrobial substances, such as lactoferrin, lysoszyme, myeloperoxidase and other strongly biologically active mediators (McArthur and Taichman, 1976; Tsai et al., 1998). The release of

lysosomal enzymes in conjunction with the formation of active oxygen metabolites causes severe destruction of the periodontal tissue.

Host responses such as the release of cytokines and other proinflammatory mediators might in turn increase the inflammation of the periodontal tissues and thus be more harmful to the host. Cytokines are low molecular weight polypeptides (5-70 kDa) (Bendtzen, 1994) and are soluble mediators that regulate or modify the activity of other cells. Recent work (Nassar et al., 2002) has demonstrated that *P. gingivalis* fimbrillin-specific peptides, lipopolysacharides (LPS), or heat-killed whole cell preparations of the putative pathogen induce the expression of host chemoattractant cytokines (chemokines). These consist of the interleukin-8 (IL-8) and the monocyte chemotactic protein 1 (MCP-1). Chemokines form a family of closely related, secreted proteins, which are specialised in the mobilisation of leukocytes to infected areas. Both IL-8 and MCP-1 are potent chemokines in directing neutrophil and monocyte migration to infected sites (Gerard and Rollins, 2001). Live cells of *P. gingivalis* abolish normal IL-8 and MCP-1 responses (Nassar et al., 2002). Other workers (Gemmell et al., 2000) have further demonstrated that *P. gingivalis*-specific T cells, monocytes and B cells produce chemokines in response to *P. gingivalis* outer membrane antigens. The periodontal pathogen *A. actinomycetemcomitans* induces the expression of IL-1α and IL-1β in gingival epithelial cells (Sfakianakis et al., 2001). Other periodontal bacteria including *Tannerella forsythensis*, *Campylobacter curvus*, *Eikenella corrodens*, *F. nucleatum*, and *Prevotella intermedia* have all been demonstrated to induce the expression of IL-8 (Han et al., 2000). The secretion of host chemokines plays an important role in promoting a leukocyte infiltrate at the site of inflammation (Mukaida et al., 1998). As an example, the IL-8 chemokine exhibits a potent chemotactic activity for neutrophils (Mukaida et al., 1998) that can phagocytose and digest bacteria. If the neutrophil however becomes overloaded with bacteria then it may degranulate, releasing toxic enzymes that further exacerbate gingival inflammation (Kinane, 2001). The extent of the host inflammatory response is critical to the disease process. Its sole purpose is to protect and prevent host cells from becoming damaged by bacteria. However, it is also detrimental as it is thought to cause much of the tissue damage that occurs in periodontal disease.

1.4.2 The microbiota associated with healthy gingivae

Studies of the microbiota of the oral cavity have demonstrated it to be diverse with up to 600 different species (Paster et al., 2001; Kazor et al., 2003), which is believed to account for 50% of the total oral microbiota (Socransky, 1963). There is very little data regarding the microbial composition of supragingival plaque and more importantly how it compares to the subgingival plaque on the same tooth surface. Previous culture-dependent studies (Slots, 1979; Tanner et al., 1979; Moore et al., 1987; Zee et al., 1996) have demonstrated the different cultivable flora that predominate in supragingival plaque of subjects with no gingivitis. These findings have been summarised in table 1.1.

PREDOMINANT MICROBIOTA OF SUPRAGINGIVAL PLAQUE IN GINGIVAL HEALTH	
GRAM POSITIVE BACTERIA	**GRAM NEGATIVE BACTERIA**
Streptococcus oralis	*Selenomonas* spp.
Streptococcus sanguinis	*Capnocytophaga* ochracea
Streptococcus anginosus	*Leptotrichia buccalis*
Staphylococcus epidermidis	*Veillonella dispar*
Actinomyces viscosus	*Veillonella parvula*
Actinomyces naeslundii	
Gemella morbillorum	
Rothia dentocariosa	

Table 1.1: Predominant cultivable microbiota sampled from supragingival plaque from subjects with no periodontal disease. Taken and adapted from (Slots, 1979; Tanner et al., 1979; Moore et al., 1987; Zee et al., 1996)

The Gram-positive cocci and rods tend to be the predominant microbiota in supragingival plaque sampled from patients with no periodontal disease (Moore et al., 1987; Zee et al., 1996). In particular, the species thought to be compatible with the healthy periodontium include members of the genera *Actinomyces*, *Streptococcus* and *Veillonella* (Moore and Moore, 1994). Periodontal pathogens can also be found in the supragingival plaque of subjects with no periodontal disease. Anaerobic culturing of supragingival plaque from periodontally healthy subjects has demonstrated a presence of periodontal pathogens such as *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *Tanerella forsythensis*, albeit at lower numbers when compared to subgingival plaque from subjects with periodontitis (van Winkelhoff et al., 2002). Other workers (Gmur et al., 1999)

have confirmed that supragingival plaque is a natural habitat for periodontitis-associated bacteria in subjects who are periodontally healthy. They also demonstrated that colonisation with *A. actinomycetemcomitans*, *T. forsythensis* and *Campylobacter rectus* is mostly stable despite above average plaque control.

1.4.3 The microbiota associated with gingivitis

The proportion of Gram-positive cocci in supragingival plaque has been observed to decrease significantly in gingivitis. The Gram-positive rods either remain stable or increase slightly in numbers, whilst the number of Gram-negative cocci and rods increase significantly with developing gingivitis (Moore et al., 1987; Moore and Moore, 1994; Rudiger et al., 2002; Zee et al., 1996). Thus there is a shift from the predominant Gram-positive cocci in healthy gingivae through to a predominant microbiota of Gram-negative cocci and rods as well as Gram-positive rods as gingivitis develops. Culture-dependent studies have documented a varied spectrum of organisms, which have been summarised in table 1.2 (Slots, 1979; Tanner et al., 1979; Page, 1986; Moore et al., 1987; Zee et al., 1996).

PREDOMINANT MICROBIOTA OF SUPRAGINGIVAL PLAQUE IN GINGIVITIS	
GRAM POSITIVE BACTERIA	**GRAM NEGATIVE BACTERIA**
Streptococcus anginosus	*Fusobacterium nucleatum*
Streptococcus sanguinis	*Prevotella intermedia*
Actinomyces israellii	*Campylobacter rectus*
Propionibacterium granulosum	*Campylobacter concisus*
Gemella morbillorum	*Veillonella dispar*
	Capnocytophaga gingivalis
	Capnocytophaga ochracea
	Eikenella corrodens

Table 1.2: Predominant cultivable microbiota sampled from supragingival plaque from subjects with gingivitis. Taken and adapted from (Slots, 1979; Tanner et al., 1979; Page, 1986; Moore et al., 1987; Zee et al., 1996)

Molecular studies have provided a broader insight into the microbial composition of supragingival plaque associated with gingivitis. Previous workers (Ximenez-Fyvie et al., 2000a) have determined the presence and levels of 40 bacterial taxa in the supragingival plaque of subjects with and without gingivitis. This was achieved using whole genomic DNA probes and checkerboard DNA-DNA hybridisation assays. The results from this study demonstrated that all 40 organisms could be detected in the supragingival plaque of both sample groups. Thus bacterial numbers, rather than richness might be the main differentiating factor between the sample groups. Other clinical studies have identified 10-15 bacterial species that are potential periodontal pathogens in adults (Haffajee and Socransky, 1994; van Winkelhoff et al., 2002; Tamura et al., 2006). Of these, the 3 most cited are *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*, which subsequently have been implicated as the main microbial aetiological agents of periodontal disease (Slots et al., 1986; Wojicicki et al., 1986; Bragd et al., 1987; Tanner et al., 2002b).

1.5 The progression of gingivitis to periodontitis

Although periodontitis is observed to precede gingivitis, not all cases of gingivitis will lead to periodontitis (Brown and Loe, 1993). The term periodontitis describes the destructive forms of periodontal diseases. Like gingivitis, it is initiated by the microbiota present in dental plaque and influenced by the immune and inflammatory response. Periodontitis is characterised by inflammation of the periodontal tissue that results in the apical migration of epithelial attachment as well as the loss of periodontal hard and soft tissue (Kinane et al., 2001). Bleeding of the gingivae following application of pressure, as well as increased mobility, drifting and/or tooth exfoliation may also occur (Flemmig, 1999). Thus periodontitis is the harshest of the periodontal diseases which ultimately may result in tooth loss (Kinane, 2001). Unlike gingivitis, periodontitis only occurs in a subset of the population (Albandar et al., 1999). Furthermore, periodontitis does not affect all teeth, but has a subject and site predilection (Kinane, 2001). It is believed that the site specificity and predilection of periodontitis may be a result of plaque retention and local factors such as crowding calculus or rough restorations (Kinane, 2001).

It has been proposed that periodontitis may progress in episodes of exacerbations and remissions, an idea which was coined as the 'burst hypothesis' (Socransky et al., 1984). The prevailing view is that periodontitis progresses in a continuous, rather than an episodic manner, with brief periods of localised exacerbation (Kinane, 2001). Periodontitis encompasses 4 groups of diseases that are not only clinically distinguishable but are believed to have both distinct aetiopathogenesis and risk factors.

1.5.1 Different types of periodontitis

1.5.1.1 Periodontitis as a result of systemic diseases

There is evidence which suggests that periodontitis may occur as a manifestation of systemic disease (figures 1.12 and 1.13). These include diabetes, haematological disorders, for example leukaemia, genetic disorders such as Down's syndrome and Papillon-Lefèvre syndrome, and viral diseases such as HIV (Kinane, 1999).

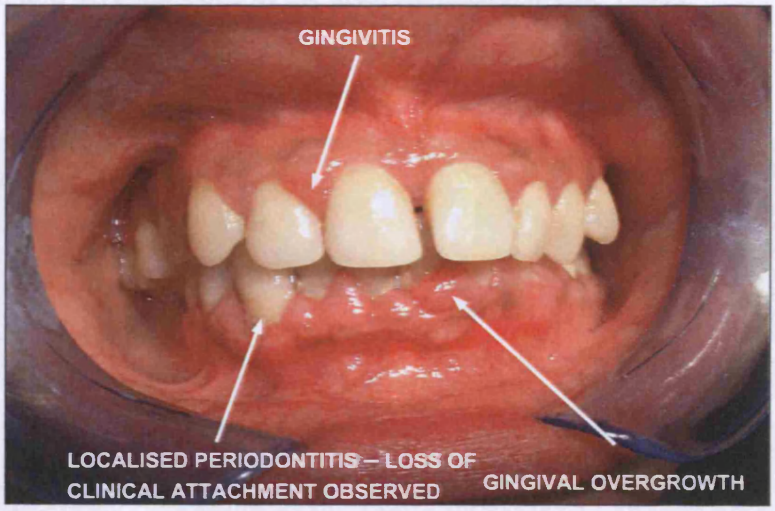


Figure 1.12: Aggressive periodontitis associated with diabetes. Clinical photograph of a diabetic patient with initial periodontitis, gingivitis and gingival overgrowth (photographs kindly provided by Luigi Nibali)

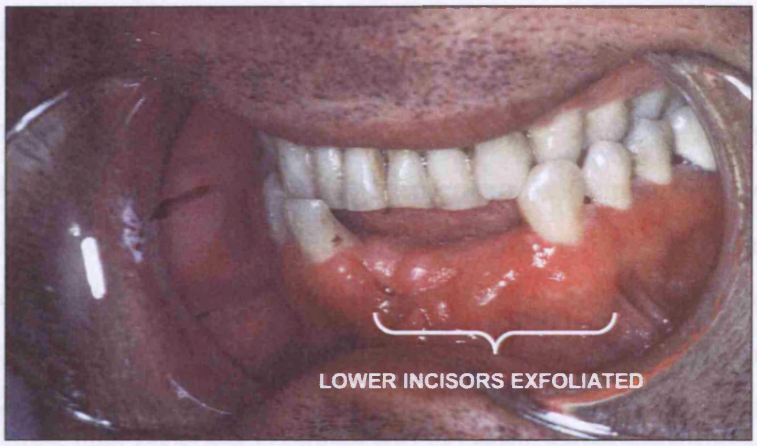


Figure 1.13: Periodontitis associated with HIV. Clinical photograph of periodontitis in a HIV patient, the lower incisors having been lost as a result of the periodontitis (photographs kindly provided by Professor Stephen Porter)

1.5.1.2 Necrotising periodontal diseases

In subjects with no known systemic disease or immune dysfunction, necrotising periodontitis (NUP) (figure 1.14) shares the same clinical and aetiological characteristics as NUG, except that patients with NUP experience loss of clinical attachment and alveolar bone at the affected sites (Novak, 1999).

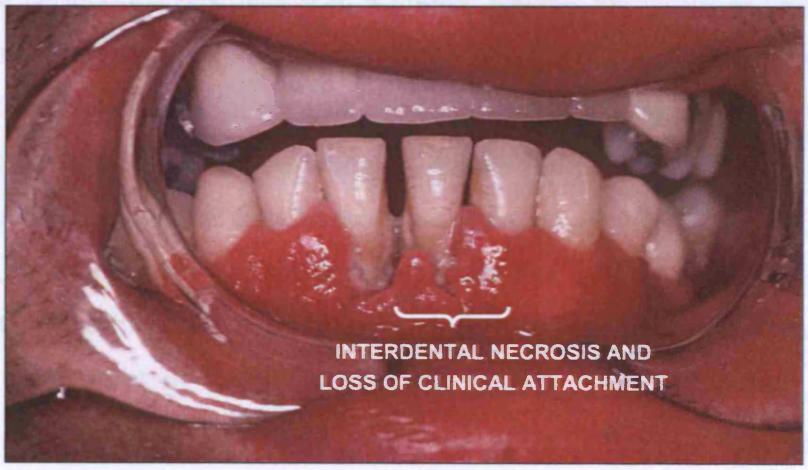


Figure 1.14: Necrotising ulcerative periodontitis (NUP). Clinical photograph of a HIV patient with NUP. The subject presents inter-dental necrosis with clear loss of clinical attachment and alveolar bone (photograph kindly provided by Professor Stephen Porter)

1.5.1.3 Aggressive periodontitis

This form of periodontitis principally affects young patients (Albandar and Tinoco, 2002). After the 1999 international workshop for a classification of periodontal diseases and conditions (The American Academy of Periodontology, 1999), the new term 'aggressive periodontitis' was adopted to replace earlier classifications that included early onset periodontitis, juvenile periodontitis and rapidly progressive periodontitis (Armitage et al., 2000; Albandar and Rams, 2002). The term 'localised aggressive periodontitis,' (LAP), replaced the older term 'localised juvenile periodontitis,' (LJP). Likewise, the new term 'generalised aggressive periodontitis,' (GAP), has roughly replaced the older term of 'generalised juvenile periodontitis' (GJP) (Armitage, 1999). The term pre-pubertal periodontitis has also been discarded and is now classified as either localised or generalised periodontitis. Although aggressive periodontitis may occur in young subjects, it is uncommon in subjects without congenital or systemic diseases. Previous workers (Albandar et al., 1997; Albandar and Tinoco, 2002) have demonstrated that despite the wide occurrence of gingivitis in children and young adults, there is a low prevalence of periodontitis in these individuals (Albandar, 2002).

Extensive periodontal destruction in a young, healthy individual is likely to be some form of aggressive periodontitis. If this periodontal destruction is localised to the interproximal areas of the first permanent molars and incisors (figure 1.15 A and B), a diagnosis of LAP is made. If the periodontal destruction is found in 3 more teeth other than the first molars and incisors (figure 1.16 A and B), then it is GAP (Armitage, 1999). This does not mean that LAP is merely a localised form of GAP as there are specific features that differentiate both forms of the disease. The onset of LAP, for example, is first diagnosed during puberty whereas GAP is usually detected under the age of 30 years although some patients may be older (Lang et al., 1999). LAP patients have a robust serum antibody response to periodontal pathogens, whereas GAP patients are said to exhibit poor antibody responses to these pathogens (Lang et al., 1999).

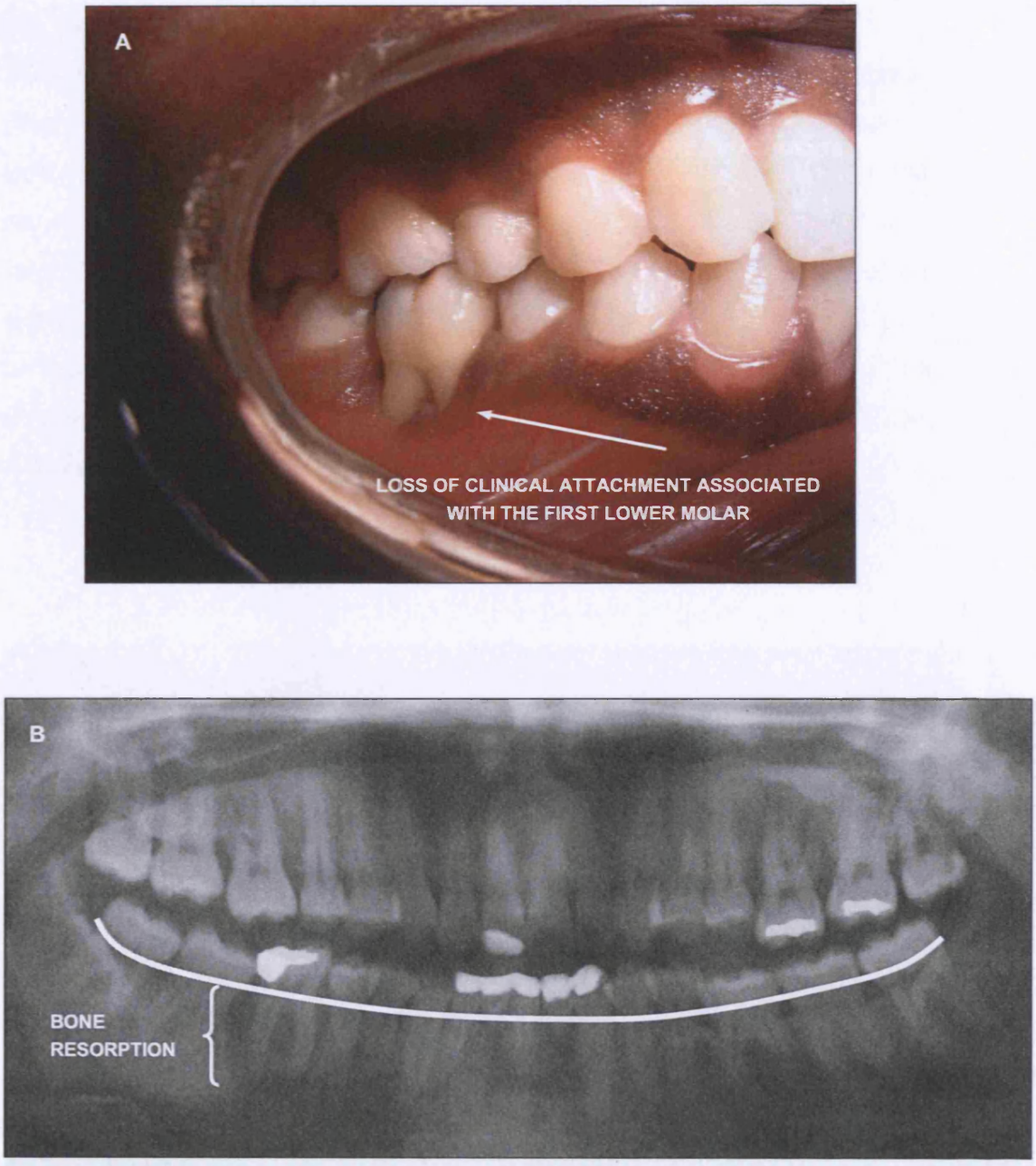


Figure 1.15: (A) Clinical photograph of local aggressive periodontitis (LAP): In a patient younger than 30 years of age, periodontitis is more pronounced on the first lower right molar. **(B) Panoramic radiograph of LAP.** Demonstrating bone resorption confined predominantly to the incisors and the first molars. The alveolar bone surrounding the first lower right molar is almost all resorbed. A white line has been inserted to approximately demonstrate where the bone should be in a healthy subject (photographs kindly provided by Luigi Nibali)

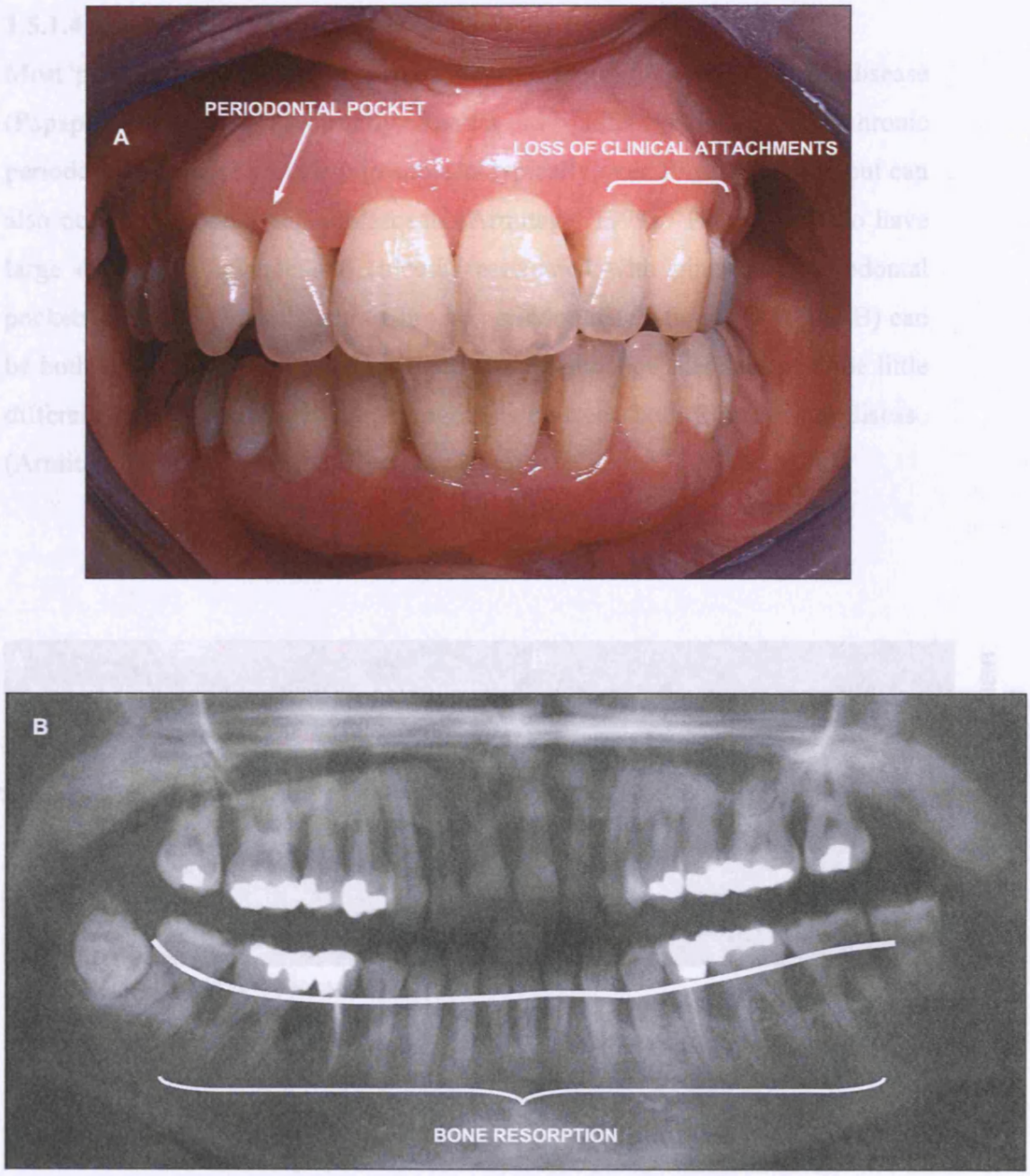


Figure 1.16: (A) Clinical photograph of generalised aggressive periodontitis (GAP): In a patient younger than 30 years of age, periodontitis in this case is not confined only to the incisors and first molars. **(B) Panoramic radiograph of GAP.** Demonstrating bone resorption associated with most teeth. A white line has been inserted to approximately demonstrate where the bone should be in a healthy subject (photographs kindly provided by Luigi Nibali)

1.5.1.4 Chronic periodontitis

Most patients with periodontitis tend to have the chronic form of the disease (Papapanou, 1996). Formerly known as 'adult periodontitis,' chronic periodontitis is more prevalent in subjects typically over 30 years of age, but can also occur in children and adolescents (Armitage, 1999). Patients tend to have large deposits of plaque and calculus associated with gingivitis, periodontal pockets and loss of attachment. Chronic periodontitis (figure 1.17 A and B) can be both generalised (GCP) and localised (LCP), although there seems to be little difference in the aetiology and pathogenesis between both forms of the disease (Armitage, 1999).

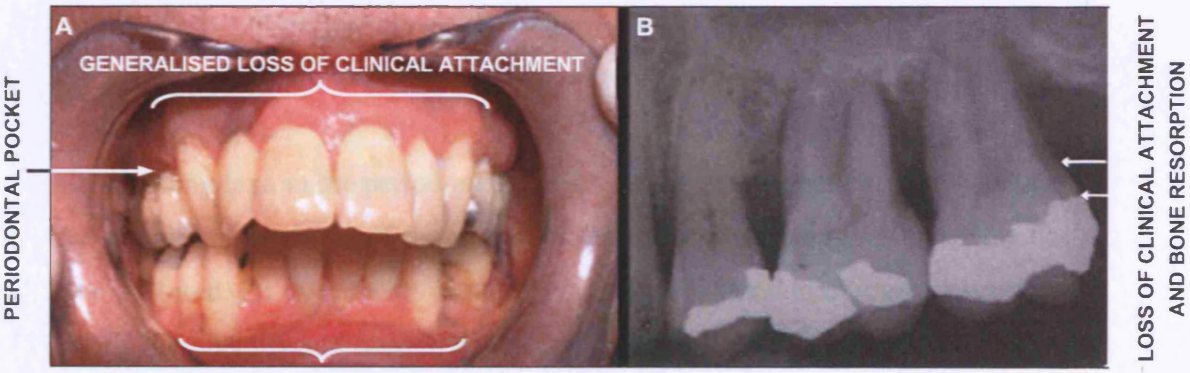


Figure 1.17: (A) Clinical photograph of generalised chronic periodontitis (GCP): In a patient older than 30 years of age, periodontitis in this case is not confined only to the incisors and first molars. **(B) Radiograph of GCP.** Demonstrating bone resorption associated with molars and pre-molars (photographs kindly provided by Luigi Nibali)

1.5.2 The main microbial aetiological agents of periodontal disease

Bacterial colonisation refers to the persistent presence of indigenous, resident or commensal bacteria in or on the host. The successful persistence or multiplication of a pathogen within a host is alternatively defined as an infection (Finlay and Falkow, 1989). Infection does not always result in disease, and conversely colonisation might sometimes lead to an opportunistic infectious disease (Asikainen and Chen, 1999). In highly polymicrobial infections such as periodontal diseases, assessing the pathogenic nature of each suspected etiological agent is a delicate, laborious task. It is difficult to say with certainty whether disease results from the colonisation of an exogenous pathogen or whether it is caused by a modification of the proportions of commensal bacteria. Other difficulties in defining which bacteria are periodontal pathogens include:

(i) When culturing from a periodontal pocket, any number ranging from 30-100 species, any of which may be a pathogen, may be recovered (Haffajee and Socransky, 1994).

(ii) Many taxa in the periodontal pocket are 'yet to be cultured.' It is thought that approximately 50% of the oral microbiota (approximately 600 species) has been cultured. It is quite probable that many periodontal pathogens also exist among the uncultivated portion of the oral microbiota (Paster et al., 2001; Kazor et al., 2003).

(iii) Opportunistic species may proliferate as a result of the disease, but not necessarily be the cause of the disease. These opportunists may proliferate in numbers concomitantly with or after the true pathogens, thus making their differentiation very difficult (Haffajee and Socransky, 1994).

(iv) Strains of periodontal pathogens may differ in virulence. Previous workers (Amano et al., 2004) have demonstrated that both disease-associated and non-disease-associated genotypes exist in *P. gingivalis*, with a significant predominance of *P. gingivalis* with type II fimA in periodontitis patients.

Koch's postulates have been extensively used, particularly in the 19th century, to identify serious pathogens. These postulates where aimed at major pathogens (usually a single pathogen per disease), but not at opportunistic pathogens, such as the periodontal pathogens and cariogenic bacteria (Nishihara and Koseki,

2004). Previous workers (Socransky, 1977) have since proposed several modification of Koch's postulates for the determination of periodontal pathogens, which are as follows:

(i) The major portion of the target bacteria should be associated with periodontitis
(ii) Elimination of target bacteria will result in stopping disease progression
(iii) Host response against target bacteria should be elucidated
(iv) Animal models (if possible) should be used to demonstrate pathogenicity
(v) Unique mechanisms of pathogenicity should be indicated

The above criteria have been used to nominate several oral taxa as true periodontal pathogens because they appear to be implicated in the perpetuation and progression of periodontal disease (Dzink et al., 1985, 1988; Haffajee and Socransky, 1994; Socransky et al., 1998; Socransky and Haffajee, 2002). Three species, *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*, are almost always strongly associated with disease status, disease progression and unsuccessful therapy (Tanner et al., 1979; Slots et al., 1986; Wojicicki C.J et al., 1986; Bragd et al., 1987; Dzink et al., 1988; Haffajee and Socransky, 1994; Haffajee et al., 1999; Ximenez-Fyvie et al., 2000b; Socransky and Haffajee, 2002). For such reasons these 3 bacteria have received considerable investigative attention and have been implicated as the main microbial aetiological agents of periodontal destruction.

1.5.2.1 *Porphyromonas gingivalis*

P. gingivalis is a short, anaerobic, Gram-negative rod (figure 1.18) which is non-sporing, assaccharolytic and non-motile (Holt et al., 1994). The organism forms brown/black colonies on blood agar (figure 1.19) as a result of protoheme production (Holt et al., 1994). The presence of *P. gingivalis*, acting either alone or as part of a mixed infection with other bacteria, appears to be essential for periodontal disease, especially in concert with the absence of beneficial species or certain host immunological deficiencies (Socransky and Haffajee, 1992; Haffajee and Socransky, 1994).

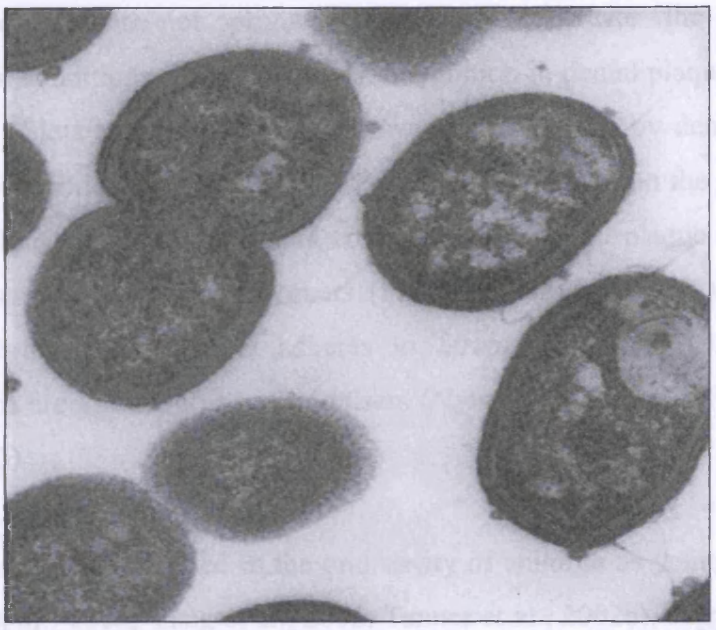


Figure 1.18: Transmission electron micrographs (x20,000 magnification) of *P. gingivalis* demonstrating its "short" rod-shaped or coccobacillary morphology (photograph kindly provided by Ms Nicky Mordan)

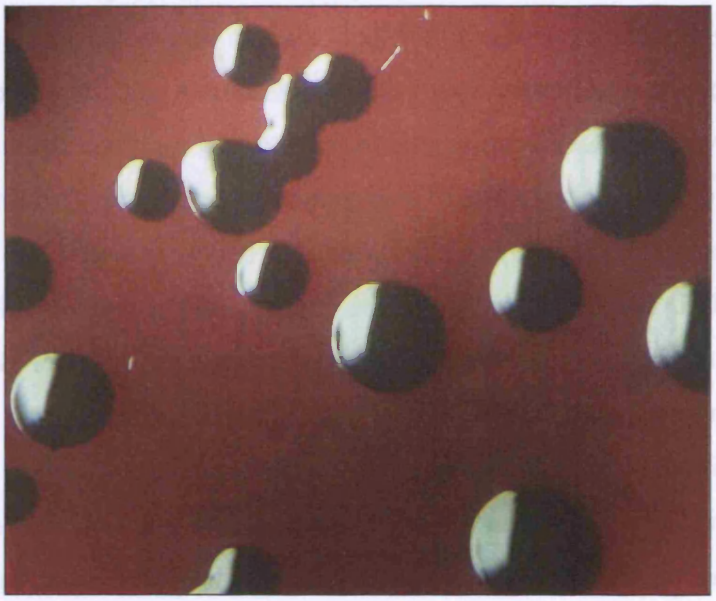


Figure 1.19: Black-pigmented colonies of *P. gingivalis*. Grown on agar supplemented with blood (photograph kindly provided by Dr Derren Ready)

P. gingivalis is usually among the secondary colonisers of the oral cavity, requiring the presence of primary colonisers to create the necessary environmental conditions for its successful integration in dental plaque biofilms. Early studies (Slots and Gibbons, 1978) have confirmed this by demonstrating that the introduction of an extraneous strain of *P. gingivalis* into the oral cavity of human volunteers resulted in rapid colonisation of dental plaque but not on clean tooth surfaces. Previous workers (Slots and Gibbons, 1978) have also demonstrated that *P. gingivalis* adheres to *Streptococcus* and *Actinomyces* species, which are known primary colonisers (Nyvad and Kilian, 1987; Palmer, Jr. et al., 2003).

P. gingivalis has been detected in the oral cavity of children as young as 1 year (McClellan et al., 1996; Yang et al., 2002; Tanner et al., 2002b), suggesting that acquisition of the pathogen may occur in the first months of life. Initial entry to the oral cavity is thought to occur by transmission from infected individuals (Greenstein and Lamster, 1997; Tuite-McDonnell et al., 1997; Rosa et al., 2002), with the sharing of saliva being considered as the most important factor for transmission of the pathogen. Although the primary ecological niche of *P. gingivalis* is the periodontal pocket and the gingival sulcus, this pathogen encounters many of the intraoral surfaces upon entry into the oral cavity. Adhesion to oral surfaces remote from the gingival sulcus allows for the pathogen to become established in that region. Further colonisation of the region occurs later as a result of cell proliferation as well as the translocation of dislodged cells to other regions within dental plaque (Slots and Gibbons, 1978; Takazoe et al., 1984). There are various adhesin molecules that not only allow *P. gingivalis* to bind to other secondary colonisers such as *F. nucleatum* (Bradshaw et al., 1998) and *T. forsythensis* (Kinder and Holt, 1989), but also facilitate the adherence to and invasion of host cells. Traditionally the term 'invasion' has been taken to mean intercellular penetration, that is to say bacteria locating between the host cells (Lamont and Yilmaz, 2002). Intracellular invasion, that is to say bacteria locating within epithelial cells, is an important pathogenic trait of some oral pathogens. The ability to invade host cells varies between different strains of *P. gingivalis* (Dorn et al., 2000). In terms of invasion of epithelial cells, two *in vitro* models have been used in order to study this phenomenon: (i)

culturing with gingival epithelial cells; (ii) transformed oral epithelial cells such as the KB cell line (Lamont and Yilmaz, 2002). The process of invasion of gingival epithelial cells has been demonstrated as being a rapid and efficient process that reaches completion after as little as 12 minutes (Belton et al., 1999). The invasive process begins with a proximal association between *P. gingivalis* and the gingival epithelial cell.

At this level of close proximity between the pathogen and gingival epithelial cells, several products synthesised by *P. gingivalis* can have detrimental effects on the host cells. Metabolic by-products, such as succinic acid produced by *P. gingivalis*, have been demonstrated to dampen the immune response by inhibiting neutrophil chemotaxis (Rotstein et al., 1985) as well as impairing host PMN responses to chemotactic peptides by depolarizing PMN membranes (Novak and Cohen, 1991). *P. gingivalis* also secretes a novel set of extracellular proteins, many of which result in the degradation of periodontal tissues (Park and Lamont, 1998). Some of these extracellular proteins include specific proteases (see section 1.4.1). In addition to providing amino acids, peptides and haemin for growth, these proteases are also believed to enable the processing of essential cell surface components and providing substrates for cell surface adhesion (Lamont and Jenkinson, 2000; Kadowaki et al., 1994). These proteases are suspected of ultimately causing enzymatic degradation of components of the periodontal tissue, such as collagen, thus leading to periodontal destruction (Uitto et al., 1990).

Adhesion between host cells and *P. gingivalis* is largely facilitated by adhesins known as fimbriae that are located on the pathogen cell surface. Fimbriae are polymers that are composed of fimbrillin subunits (Dickinson et al., 1988). Previous workers have reported that this adhesin not only allows for interspecies coaggregation but also enables adhesion to host epithelial/endothelial cells and subsequently might lead to invasion (Dorn et al., 2000; Yilmaz et al., 2002). Furthermore, fimbriae have also been reported to adhere to saliva-coated hydroxyapatite (Lee et al., 1991), red blood cells (Ogawa and Hamada, 1994), monocytes and macrophages (Ogawa et al., 1994) and gingival fibroblasts (Hanazawa et al., 1988). Fimbriae attach to cognate receptors on the gingival

epithelial cell surfaces, which are primarily β1 integrins (Weinberg et al., 1997; Yilmaz et al., 2002). Another group of adhesin proteins synthesised by *P. gingivalis* are the hemagglutinins, which when expressed may promote colonisation of host cells by mediating the binding of the pathogen to host receptors (Lamont and Jenkinson, 1998). Adhesion can lead to rearrangement of epithelial cell cytoskeletal components, namely both actin microfilaments and tubulin microtubules, and it is this which facilitates bacterial entry into host cells (Finlay and Cossart, 1997; Lamont and Jenkinson, 1998). An altering of calcium ion fluxes (Izutsu et al., 1996) is also required for invasion. These calcium fluxes are likely to be important in many signalling events that may converge on calcium-gated channels on the cytoplasmic membrane (Lamont and Jenkinson, 1998).

Once *P. gingivalis* locates itself intracellularly it may then become protected from the host immune response (Kagnoff and Eckmann, 1997), interrupt aspects of epithelial cell function such as cytokine synthesis (Kagnoff and Eckmann, 1997) and down-regulate the expression of chemokines, such as interleukin IL-8 (Darveau et al., 1998). The invading *P. gingivalis* has also been demonstrated to provoke a limited cleavage and activation of several host MMPs (Ding et al., 1995) as well as modulating mitogen-activated protein (MAP) kinases (Watanabe et al., 2001) that are involved in cytokine and stress responses (Robinson and Cobb, 1997). The entrance of *P. gingivalis* into host cells is also believed to contribute to bacterial persistence and periodontal disease progression (Rudney et al., 2001). Adherence structures and mechanisms of this organism are significant virulence factors in the progression of periodontal disease. Furthermore, the ability of *P. gingivalis* to produce harmful enzymes and toxins has been documented to impart detrimental effects on host periodontal tissues and results in alveolar bone loss. This is an important feature of advanced periodontal disease (Lamont and Jenkinson, 1998).

There is accumulating evidence which suggests that *P. gingivalis* is a member of the commensal flora of the oral cavity (Loos et al., 1993; Menard and Mouton, 1995; McClellan et al., 1996). These findings do not necessarily weaken the evidence for a decisive role of *P. gingivalis* in periodontal diseases. To play a

role in multifactorial, polymicrobial infections such as periodontal disease, *P. gingivalis* should demonstrate specific properties allowing it to shift from commensal to pathogenic action. Such properties may imply molecular interactions between the organism and a key target of the periodontium, the gingival epithelial cell. Examples of these molecular interactions include the activation as well as down-regulation of MMPs (Ding et al., 1995; Fravalo et al., 1996). A community analysis of dental plaque might help demonstrate any oral taxa associated with the prevalence of this pathogen in subjects with and without periodontal disease. Furthermore, such studies might also help to determine whether the presence of other oral bacteria may modulate the pathogenic potential of *P. gingivalis*.

1.5.2.2 *Actinobacillus actinomycetemcomitans*

A. actinomycetemcomitans is a Gram-negative, non-motile, saccharolytic, capnophilic, facultatively anaerobic coccobacillus (figure 1.20) in the γ subdivision of the Proteobacteria (Alaluusua and Asikainen, 1988; Kachlany et al., 2001). As a member of the Pasteurellaceae, it is related to the *Haemophilus* and *Pasteurella* genera. Its species name is derived from the fact that this pathogen was first isolated from actinomycotic oral lesions along with *Actinomyces israellii* (Holmes et al., 1999). The organism forms 'crinkled, rough' colonies on solid medium with a characteristic star-like structure as the colonies become older (figure 1.21). Repeated subculturing results in the disappearance of this star shape morphology, resulting in colonies that are both smooth and opaque. This transformation is believed to be associated with the loss of fimbriae (Inouye et al., 1990).

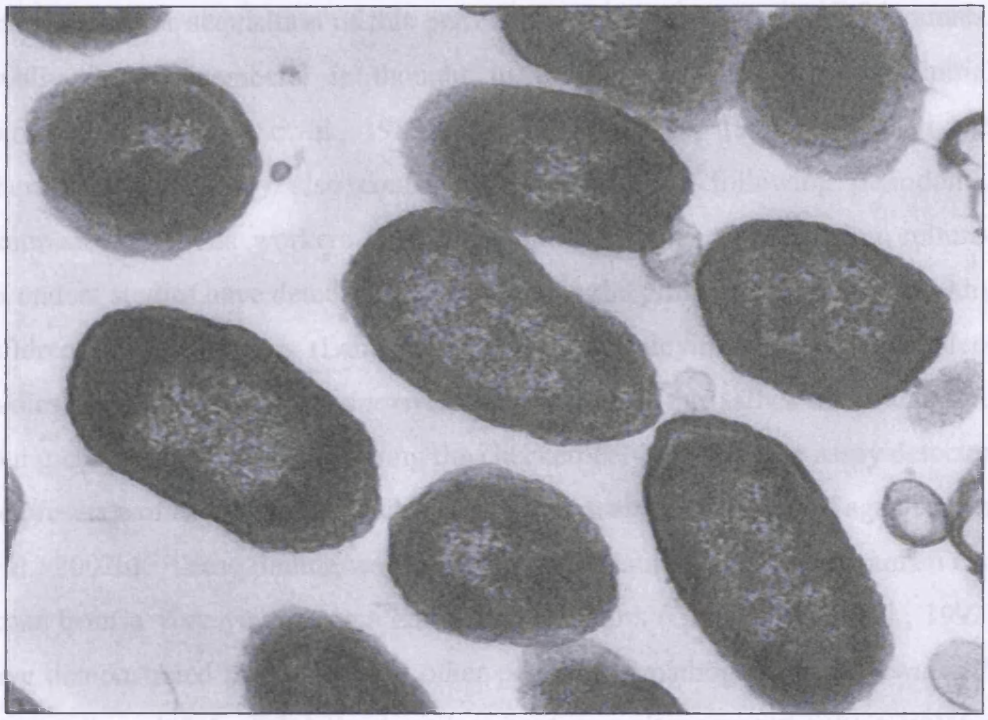


Figure 1.20: Transmission electron micrographs (x20,000 magnification) of *A. actinomycetemcomitans* demonstrating its rod-shaped morphology (photograph kindly provided by Ms Nicky Mordan)

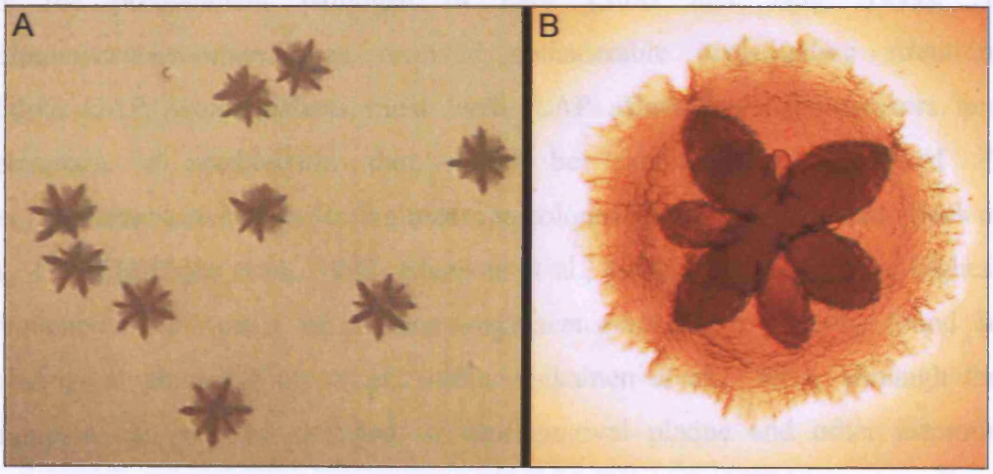


Figure 1.21: (A) Star-like morphologies of multiple colonies. (B) Crinkled-rough surface of *A. actinomycetemcomitans* colony. A single colony of *A. actinomycetemcomitans* demonstrating a 'crinkled-rough' surface as well as the characteristic star shape (photograph kindly provided by Dr Derren Ready)

Transmission or acquisition of this periodontal pathogen from other individuals, usually family members, is thought to be the possible source of initial colonisation (Zambon et al., 1983; Troil-Linden et al., 1995; Asikainen and Chen, 1999) and may also contribute in re-infection following periodontal treatment. Previous workers (Alaluusua and Asikainen, 1988) using culture-dependent studies have detected this pathogen in the primary dentition of healthy children. Other workers (Lamell et al., 2000) employing culture-independent studies have detected *A. actinomycetemcomitans* in the saliva of infants and young children. Recent work using the checkerboard DNA probe assay detected the presence of this pathogen in children as young as 6-18 months of age (Tanner et al., 2002b). These finding would suggest that acquisition of this organism can occur from a very young age. Earlier investigators (Christersson et al., 1992) have demonstrated that unlike the other periodontal pathogens *P. gingivalis*, *P. intermedia* and *T. forsythensis*, *A. actinomycetemcomitans* occurs only in isolated sites. This has been further confirmed by other studies (Beck et al., 1992; Haffajee et al., 1992; Ebersole et al., 1994) in which *A. actinomycetemcomitans* was observed colonising fewer sites than other pathogens such as *P. gingivalis*.

As the predominant pathogen in LAP (Slots and Ting, 1999), *A. actinomycetemcomitans* has received considerable investigative attention. Unlike GAP, which affects most teeth, LAP affects only the incisors and premolars, a predilection that might be explained in terms of *A. actinomycetemcomitans* selective nature in colonising only specific sites (Beck et al., 1992; Haffajee et al., 1992; Ebersole et al., 1994). In humans, the highest frequencies and levels of *A. actinomycetemcomitans* are usually found in subgingival plaque (Slots et al., 1980; Asikainen et al., 1991), although the organism can also be detected in supragingival plaque and other intraoral surfaces (Slots et al., 1980; Slots et al., 1990; Muller et al., 1993).

Both secreted and cell wall components of *A. actinomycetemcomitans* are mitogenic to host B cells (Nishihara et al., 1987) and also promote cytokine induction from other host cells (Jiang and Graves, 1999). *A. actinomycetemcomitans* can produce two immunomodulatory toxins that inhibit such host immune responses (Henderson et al., 2003). These are (i) the

leukotoxin, a member of the family of repeat in toxin (RTX) toxins (Lally et al., 1999) and (ii) the cytolethal distending toxin (CDT) (Henderson et al., 2003). The *A. actinomycetemcomitans* leukotoxin binds to the cell surfaces of lymphocytes and granulocytes on cognate receptors belonging to a member of the β2-integrin family, LFA-1 (Lally et al., 1997), and kills them (Lally et al., 1999). It is assumed that this enables *A. actinomycetemcomitans* to evade an important part of the innate host immune system. The majority of *A. actinomycetemcomitans* isolates produce bioactive CDT (Ahmed et al., 2001), which is a heat-labile cytotoxin (Shenker et al., 2001). This toxin is coded for by the CDT encoding operon (cdtABC) (Mayer et al., 1999). Previous investigators have reported that CdtB impairs lymphocyte activity and can also bring about cell cycle arrest and apoptosis of human T-cells (Shenker et al., 2001). Another potential virulence factor of *A. actinomycetemcomitans* has the ability to induce the secretion of host MMPs such as collagenases (Fives-Taylor et al., 1999), which are considered to be key initiators of collagen degradation during the progression of periodontal tissue destruction (Birkedal-Hansen, 1988; Sorsa et al., 1992).

Intracellular invasion of *A. actinomycetemcomitans* was first reported using KB cells (Fives-Taylor et al., 1999). This multi-step process commences with the pathogen adhering to a transferrin receptor (Meyer et al., 1997), although binding to integrins may also provide a secondary means of entry (Meyer et al., 1997). Much in the same way as *P. gingivalis*, the invasion into epithelial cells requires the remodelling of actin microfilaments (Fives-Taylor et al., 1995). Other workers have demonstrated that other strains of this pathogen can also invade KB epithelial cells via actin-independent receptor-mediated endocytosis (Brissette and Fives-Taylor, 1999). Internalised *A. actinomycetemcomitans* is initially confined within a host-derived membrane vacuole, or endosome. These endosomes are subsequently broken down, possibly by the secretion of phospholipase C (Lamont and Yilmaz, 2002), thus allowing the bacteria to reside in the cytoplasm (Sreenivasan et al., 1993b). It has been documented that once internalised in host cells, *A. actinomycetemcomitans* can also move into adjacent cells (Fives-Taylor et al., 1999; Lamont and Yilmaz, 2002).

Previous research analysing *A. actinomycetemcomitans* isolates from various infections has led to the suggestion that this organism might be an opportunistic pathogen (Poulsen et al., 1994; Haubek et al., 1997). Differences in virulence have been suggested to explain the occurrence of this bacterium in diseased as well as healthy periodontal sites (Hritz et al., 1996). Support for this suggestion is partly substantiated by the fact that the expression of leukotoxin varies significantly among *A. actinomycetemcomitans* strains (Hritz et al., 1996). This has been observed with the highly virulent *A. actinomycetemcomitans* JP2 strain, which is reported to express high levels of leukotoxin as opposed to other, less virulent strains of the pathogen (Brogan et al., 1994). Other workers (Johansson et al., 2000) have demonstrated that other oral bacteria , namely *P. gingivalis* and different species of *Prevotella* can modify the leukotoxicity of *A. actinomycetemcomitans*. A higher prevalence of these other pathogens at certain sites might explain the tendency for *A. actinomycetemcomitans* to colonise other specific sites. The current literature does not explain why *A. actinomycetemcomitans* favourably colonises specific teeth. Community analysis of dental plaque combined with prevalence data for this pathogen may help to further elucidate whether other oral bacteria might influence the colonisation capability of *A. actinomycetemcomitans*.

1.5.2.3 *Tannerella forsythensis*

T. forsythensis, formerly known as *Bacteroides forsythus* (Sakamoto et al., 2002), is an anaerobic, Gram-negative, non-motile rod (figure 1.22), which is normally isolated from the oral cavity together with *Campylobacter rectus* and *F. nucleatum* (Jousimie-Somer et al., 1999). This pathogen forms small, white colonies on blood agar supplemented with N-acetylmuramic acid (NAM) (figure 1.23), which is required for proliferation and maintenance of cell shape (Wyss, 1989). Previous workers have reported that this pathogen feeds on other bacteria such as *F. nucleatum* and *S. sanguinis* in order to obtain NAM (Wyss, 1989).

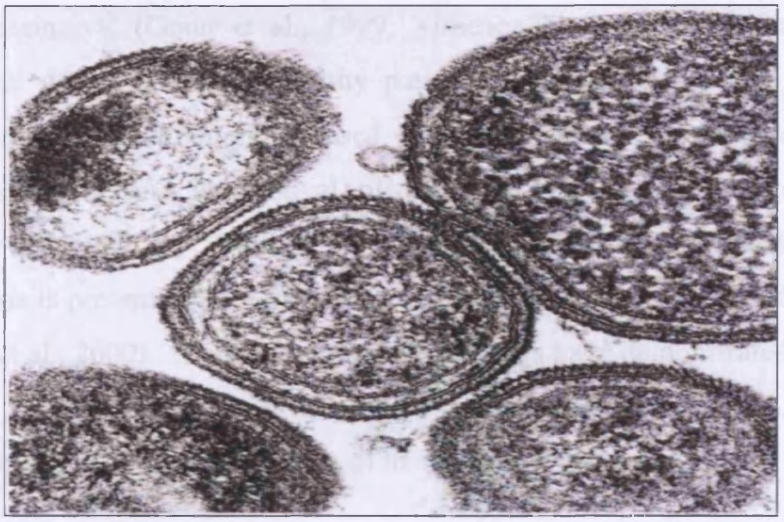


Figure 1.22: Transmission electron micrographs of *T. forsythensis* demonstrating its rod-shaped morphology (photograph kindly provided by Dr S-W. Lee)

Figure 1.23: Colony morphology of *T. forsythensis.* Small white colonies of *T. forsythensis* growing on agar supplemented with blood and NAM (photograph kindly provided by Dr Derren Ready)

Familial transmission of this pathogen has been suggested by previous workers (Umeda et al., 2004; Okada et al., 2004). *T. forsythensis* has been isolated in large numbers from periodontal pockets of patients with active periodontitis, as well as patients with early periodontal lesions (Dzink et al., 1985, 1988; Gmur et al., 1989). Other workers have also demonstrated that this pathogen is present in

both supragingival (Gmur et al., 1999; Ximenez-Fyvie et al., 2000a) and as subgingival dental plaque of healthy patients (Ximenez-Fyvie et al., 2000a; Paster et al., 2001), although increased proportions have been detected in both the supragingival and subgingival plaque from subjects with periodontitis (Ximenez-Fyvie et al., 2000a). Previous work has demonstrated that *T. forsythensis* is present in both the permanent as well as primary teeth of children (Kamma et al., 2000). Moreover, other investigators have demonstrated that this pathogen is present in children as young as 18 months (Tanner et al., 2002b), thus suggesting that early acquisition of this pathogen is possible.

The microbial pathogenesis of *T. forsythensis* has yet to be fully determined, as the putative virulence factors involved in the pathogenicity of this organism need to be adequately identified and characterised. Nevertheless, several virulence factors including a trypsin-like protease (related to gingipains of *P. gingivalis*) (Loesche et al., 1990; Saito et al., 1997) and a sialidase (Moncla et al., 1990) have been previously reported. *T. forsythensis* has also been reported to adhere to red blood cells, PMNs and fibroblasts (Munemasa et al., 2000). Other workers (Sharma et al., 1998; Honma et al., 2001) have also demonstrated that the presence of a cell surface-associated protein, BspA, is associated with binding to fibronectin. Recently, the S-layer of *T. forsythensis* has also been demonstrated to mediate haemagglutination as well as adherence of this pathogen to host cells (Sabet et al., 2003). These workers have further demonstrated that *T. forsythensis* can invade KB cells (figure 1.24 A), although the mechanisms involved are poorly understood. While adherence to KB cells is facilitated by haemagglutinins, it remains unclear whether other adhesins are involved in pathogen-to-host cell adhesion. It is also unclear whether invasion requires the remodelling of the host cells cytoskeleton, as is the case with *P. gingivalis* and *A. actinomycetemcomitans.* The invasive mechanism might be further mediated by the pathogen's S-layer (Sabet et al., 2003). Once these pathogens invade KB cells, they become surrounded by endosomes (personal communications, S-W. Lee). The invasive cells were observed to locate themselves near the nucleus of the KB cells (figures 1.24 B).

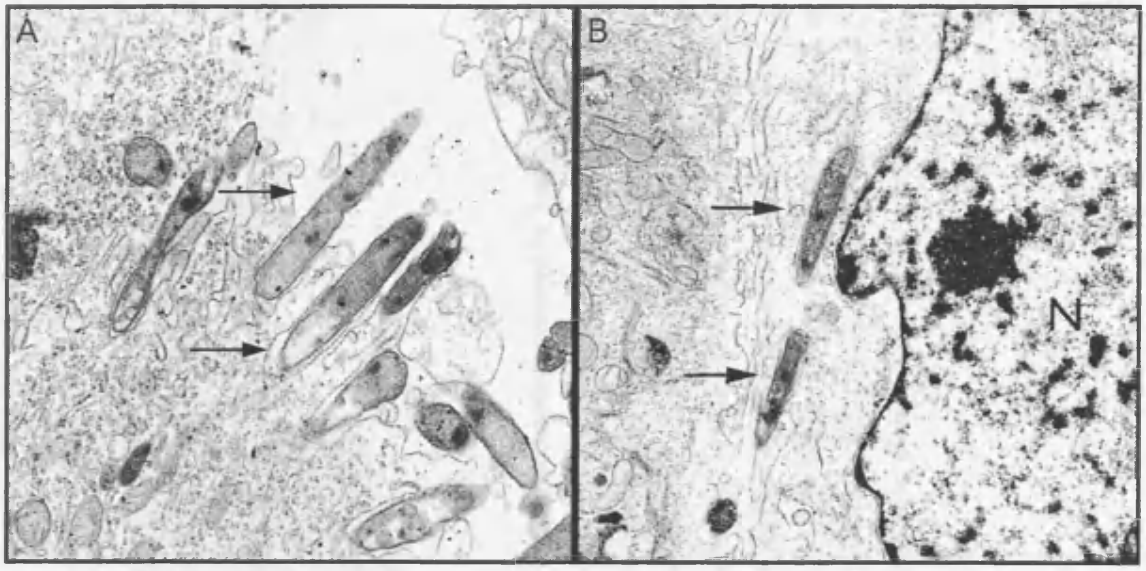


Figure 1.24: (A) Adherence of *T. forsythensis* to KB cells. Transmission electron micrograph showing adherence of *T. forsythensis* to KB cell surfaces. **(B) Invasion of *T. forsythensis* into KB cells.** Transmission electron micrograph demonstrating invasion of KB cells by *T. forsythensis*. The invading *T. forsythensis* cells are shown by arrows. N denotes the nucleus of the KB cell (photograph kindly provided by Dr S,-W. Lee)

There is little data for the virulence properties of *T. forsythensis* and its role in periodontal diseases compared to *P. gingivalis* and *A. actinomycetemcomitans*. This is because of the difficulty of cultivating *T. forsythensis* in the laboratory (Takemoto et al., 1997). This organism has nevertheless been isolated at higher frequencies and numbers in subjects with periodontal disease (Grossi et al., 1994; Kamma et al., 2001; Kamma et al., 2004) and has been implicated as one of the main microbial aetiological agents in periodontitis (Zambon, 1996). This organism has been frequently isolated with *P. gingivalis* and *T. denticola*, which indicates a possible ecological relationship between both pathogens (Takemoto et al., 1997; Ximenez-Fyvie et al., 2000a). Community analysis of dental plaque combined with prevalence data for this pathogen may help to further elucidate whether other oral bacteria may share an ecological relationship with *T. forsythensis*, or influence its prevalence within plaque biofilms.

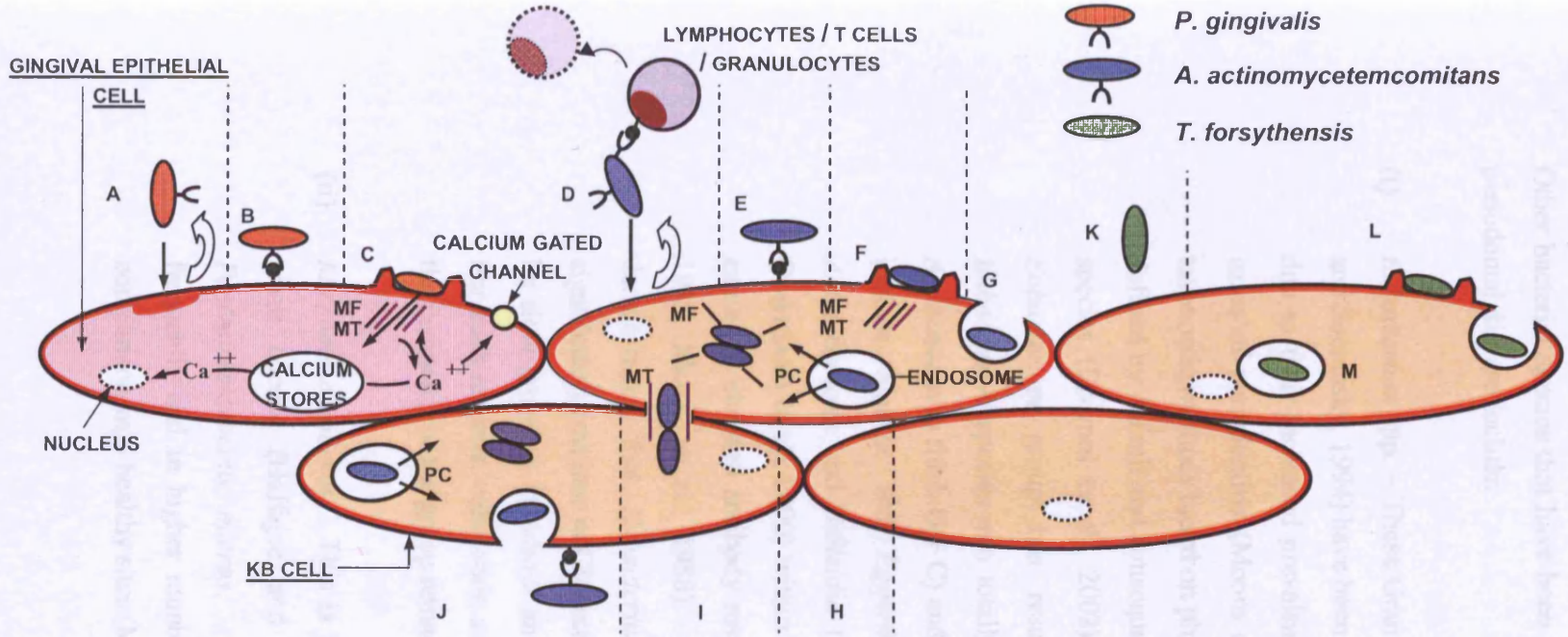


Figure 1.25: Schematic representation of the mechanisms of cell invasion. Schematic representation of the postulated cell invasion strategies used by 3 major periodontal pathogens. (A) *P. gingivalis* secreted proteins causes the release of nutrients from epithelial cells; (B) Adhesion of fimbriae to receptors, primarily β_1 integrin; (C) Microtubules (MT) and microfilaments (MF) rearrangement facilitates the invagination of gingival epithelial cell membranes resulting in the engulfment of *P. gingivalis*. Calcium ions released from intracellular stores are likely to be involved in signalling events; (D) Secreted/cell surface components from *A. actinomycetemcomitans* attract a host immune response, LtxA and the cytolethal distending toxins can degrade these; (E) Adhesion to transferrin receptors; (F) MT and MF rearrangement facilitates the invagination of gingival epithelial cell membranes resulting in (G) the engulfment of *A. actinomycetemcomitans* into the host cell which then becomes internalized within a membrane vesicle (endosome); (H) Pathogen destroys vesicle membrane by possibly secreting phospholipase C (PC); (I) Pathogen replicates and become localised at membrane protrusions through which they enter adjoining epithelial cells in a MT-dependant process; (J) Invasion can also be actin-independent; (K) Adherence of *T. forsythensis* to KB cell, pathogen S-layer is believed to be involved; (L) Invagination of epithelial cell membrane, facilitating the engulfment of the pathogen that then becomes internalised within a membrane vesicle; (M) Pathogen locates near to the nucleus of KB cells

1.5.2.4 Other microbial aetiological agents of periodontal disease

Other bacterial species that have been proposed as microbial etiological agents of periodontal disease include:

(i) *Eubacterium* spp. – These Gram-positive, obligate anaerobic rods (Haffajee and Socransky, 1994) have been suggested as possible periodontal pathogens due to their increased prevalence in diseased sites, particularly in severe cases of periodontitis (Moore et al., 1985). Prior to the introduction of taxonomic methods based on phylogeny, the genus *Eubacterium* was largely defined by default and consequently was comprised of many heterogeneous species (Downes et al., 2002). This phylogenetic diversity within the *Eubacterium* group has resulted in the re-classification of former *Eubacterium* species into totally new genera both within the phyla of the *Actinobacteria* (high G + C) and *Firmicutes* (low G + C). Examples of these include *Slackia* and *Eggerthella* (Wade et al., 1999) within the *Actinobacteria* and *Bulleidia* (Downes et al., 2000) and *Mogibacterium* (Nakazawa et al., 2000) within the *Firmicutes*. Some *Eubacterium* species can elicit elevated antibody responses in periodontitis subjects (Tew et al., 1985; Martin et al., 1988). Earlier workers (Han et al., 1991) have demonstrated that *Eubacterium* species were the only organisms to significantly increase in Chinese LAP subjects. *Eubacterium* species tend to be slow growing, fastidious and generally unreactive in biochemical tests. For such reasons, cultivation and identification of isolates are difficult and the taxonomy of the group remains indifferent (Downes et al., 2001).

(ii) *Micromonas micros* – This is a Gram-positive, anaerobic, assaccharolytic, small coccus (Haffajee and Socransky, 1994) previously known as *Peptostreptococcus micros*. This organism has been detected more frequently and in higher numbers at sites with periodontal destruction in comparison with healthy sites (Moore et al., 1985).

(iii) *Streptococcus intermedius* – This is a Gram-positive facultatively anaerobic, coccus (Tanner et al., 1994). *S. intermedius* colonises supragingival and subgingival plaque (Whiley et al., 1992) and has been detected more frequently in periodontitis subjects than in healthy subjects (Haffajee and Socransky, 1986, 1994; Kamma et al., 1994).

(iv) *Campylobacter* spp. – These are Gram-negative, microaerophilic, assaccharolytic organisms that may be straight, vibrioid (curved) or helical. Most species are motile by means of a single polar flagellum, whereas others may lack flagella and only show twitching motility (Tanner et al., 1994). *C. rectus* (formerly known as *Wolinella recta*) is commonly found in the periodontal pocket and is highly prevalent in subjects with gingivitis and periodontitis (Moore et al., 1987; Dzink et al., 1988; Macuch and Tanner, 2000; Kamma et al., 2004). Previous workers (Gillespie et al., 1992, 1993) demonstrated that *C. rectus* produces a cytotoxin that has leukotoxic activity in that it destroys PMNs. *Campylobacter showae* has also been detected more frequently and in higher numbers in subjects with periodontitis and thus may also be associated with periodontal disease (Macuch and Tanner, 2000).

(v) *Capnocytophaga* spp. – These are a group of facultative, Gram-negative fusiform rods, which require CO_2 for growth (capnophilic) and hence their name (Darby and Curtis, 2001). The *Capnocytophaga* species have been associated with periodontal disease in a number of studies (Murayama et al., 1982; Han et al., 1991; Lopez et al., 1995). Furthermore, total counts for different *Capnocytophaga* spp have been reported to be present in higher numbers in diabetes mellitus periodontitis subjects as opposed to non-diabetes mellitus subjects (Ciantar et al., 2005).

(vi) *Eikenella corrodens* – This is a Gram-negative, capnophilic, assaccharolytic small rod with blunt ends (Haffajee and Socransky, 1994) which is more

frequently isolated from sites with periodontal destruction in comparison to healthy sites (Savitt and Socransky, 1984). Recent work (Suda et al., 2002) has demonstrated that *E. corrodens* is prevalent in high numbers in the subgingival plaque of subjects with aggressive periodontitis, thus suggesting that it may be implicated in the occurrence and/or progression of periodontitis in young patients.

(vii) *Fusobacterium nucleatum* – This anaerobic, spindle-shaped rod stains negative in the Gram test, yet phylogenetically is considered to be a Gram-positive bacterium (Tanner et al., 1994). *F. nucleatum* is highly prevalent in both subjects suffering from NUG (Gmur et al., 2004) as well as periodontitis (van Winkelhoff et al., 2002). This organism has been demonstrated to induce powerful MMPs from host cells (Uitto et al., 2005), and produces tissue irritants such as butyric acid (Roberts, 2000). There is also evidence suggesting that *F. nucleatum* is immunosuppressive as it has been shown to inhibit B- and T-cell functions (Demuth et al., 1996) as well as inducing apoptotic cell death of monocytes and PMNs (Jewett et al., 2000).

(viii) *Prevotella intermedia* – This Gram-negative, anaerobic bacilli is the second black-pigmented organism to receive considerable attention after *P. gingivalis*, and appears to share similar virulence properties (Haffajee and Socransky, 1994) except it produces less gingipain activity (Jousimies-Somer et al., 1999). There is evidence suggesting a greater prevalence of this organism in subjects with NUG (Loesche et al., 1982; Gmur et al., 2004). Elevated serum antibodies to this species have also been observed in patients with periodontitis (Haffajee et al., 1988).

(ix) *Selenomonas* spp. – These are Gram-negative, curved, saccharolytic rods (Haffajee and Socransky, 1994). Previous work (Tanner et al., 1998) has demonstrated that *Selenomonas noxia* is a major species in the progression

from periodontal health to periodontal destruction. Other species such as *Selenomonas sputigena* have an increased prevalence in subjects with NUG (Gmur et al., 2004).

(x) *Spirochetes* – All oral *spirochetes* are classified in the genus *Treponema*, which are motile helical rods with tight or irregular spirals (Tanner et al., 1994). These are obligate anaerobes and tend to be nutritiously fastidious, many of which can not be cultured in the laboratory (Tanner et al., 1994). Only four species of the oral *spirochetes* have been cultured widely and reliably by several laboratories: *T. denticola*, *T. pectinovorum*, *T. socranskii* and *T. vincentii* (Chan and McLaughlin, 2000). *T. denticola* tends to be detected more frequently in subjects with periodontitis and is more prevalent in subgingival plaque than supragingival plaque (Simonson et al., 1988; Riviere et al., 1992; Takeuchi et al., 2001). Like *T. denticola*, *T. socranskii* is also detected more frequently in subjects suffering from periodontitis (Takeuchi et al., 2001). Pathogenic oral *spirochetes* have also been detected more frequently in patients with necrotising forms of periodontal destruction (Loesche et al., 1982; Novak, 1999; Gmur et al., 2004).

The pathogenicity of periodontal disease is undoubtedly complex and involves multiple bacteria with diverse virulence factors that interact with a range of host cells and immune responses. It is evident that an elevated presence of proteolytic pathogens such as *P. gingivalis* and *T. forsythensis*, or leukotoxic pathogens such *A. actinomycetemcomitans* in the gingivae would be detrimental for the health status of the host. Close proximity of these pathogens to host cells allows for destructive products such as proteases, leukotoxin and LPS, to damage the structural integrity of the periodontal tissue (Lamont and Jenkinson, 1998). Intracellular invasion by periodontal pathogens, such as *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* (summarised in figure 1.25) can also pose an equally destructive threat to the periodontal tissues. The host response to microbial aetiological agents can

also further exacerbate the extent of tissue damage, thus making periodontal diseases a host-microbial dependent condition (Taichman et al., 1977; Seow et al., 1992).

1.6 Detection of periodontal pathogens in dental plaque

1.6.1 Traditional microbiological identification

Traditional microbiological methods for the identification of bacteria require culturing on to solid or liquid media followed by a large number of diverse and complex biochemical tests. Early microscopy studies have shown that approximately 50% of the oral microbiota is unculturable (Socransky, 1963), thus conventional culturing of samples excludes the characterisation of half the oral microbiota. It is conceivable that unculturable or 'yet to be cultured' taxa which are present in dental plaque might also be playing a role in the inception and progression of periodontal diseases. The precise role which these organisms play in periodontal disease may not be determined without their identification and characterisation. These unculturable taxa fall into two broad categories:

(i) Bacteria that require nutrients or other essential components, which conventional sampling techniques, transport conditions and laboratory media do not provide e.g. sensitivity to oxygen (i.e. obligate anaerobes) or the absolute requirement for products provided by other taxa in dental plaque. These taxa are therefore broadly unknown and have been characterised only microscopically.

(ii) Well characterised taxa that are commonly observed microscopically but which for some reason cannot be cultured (Xu et al., 1982).

Traditional microbiological identification of this unculturable flora is precluded by the fact that these taxa must be grown before they can be identified and indeed characterised. A number of studies of the microbiota associated with gingivitis have been carried out using culturing techniques (Slots et al., 1978; Moore et al., 1987). Yet studies employing molecular techniques have demonstrated that culture-

dependent approaches may severely underestimate the bacterial richness in most environments (Socransky, 1963; Amann et al., 1995).

1.6.2 Molecular identification techniques for culture-dependent studies

The application of polymerase chain reaction (PCR) and DNA sequencing has markedly revolutionised the detection and identification of bacteria. The DNA from bacterial isolates can be amplified and the sequences generated can be used to identify the organisms to genus or species level. PCR is a technique that uses a DNA polymerase to generate a huge number of copies of any given section of DNA or gene (Brown, 1995). The particular stretch of DNA to be amplified, the target sequence, is identified by a pair of specific oligonucleotide DNA primers that designate the outer limits of the amplification product, the amplicon. The use of the nucleotide sequence data from 16S ribosomal RNA (16S rRNA) gene (among many others) has enabled organisms to be identified (Lane, 1991). Traditionally, the 16S rRNA gene has been selected as a candidate molecule for a variety of reasons. Principally, it is present in all bacteria and performs the same function. Its sequence is highly conserved, containing regions of conserved, variable as well as hypervariable regions. Lastly, the 16S rRNA gene is of sufficient size (ca. 1500 bases) to be easily sequenced, but still contains sufficient information for identification and phylogenetic analysis (Clarridge, III, 2004). Generally, the comparison of 16S rRNA gene sequences allows for the differentiation between organisms at the genus level across all major phyla of bacteria. In addition, it also allows for the classification of bacteria to species and sub-species levels. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relates to several species that have the same or very similar sequences (Clarridge, III, 2004). The nucleotide sequence obtained by PCR-sequencing a bacterial isolate can be compared to online databases, such as the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). This database provides over 124,000 aligned bacterial small subunit rRNA sequences and allows for a nearest neighbour, to genus or species level, to be ascribed to the particular sequence being analysed.

1.6.3 Molecular identification techniques for culture-independent studies

PCR does not only allow for the identification of culturable bacteria but also permits for the detection and identification of bacteria without the need to cultivate them. It is possible to identify bacterial species of interest, from a mixed community sample such as dental plaque, by using species-specific primers on a DNA extract of the sample itself (Tran and Rudney, 1996). This technique has been instrumental in the analysis of yet to be cultured organisms. PCR-sequencing has helped to further our understanding of how uncultivated organisms such as the bacterial division TM7 may play a role in the multifactorial process leading to periodontitis (Brinig et al, 2003).

1.7 Microbial community analysis

To adequately study microbes in complex microbial communities, novel techniques that enable the analysis of microbes at the community level without prior isolation (i.e., more conventional single-cell and pure culture studies) are required. All investigators sampling natural communities, such as dental plaque, encounter the same problem as to how well a sample reflects a community's 'true' richness and diversity. Through continued efforts to cultivate oral bacteria, it is now believed that only 50% of the total flora in the mouth has been grown (Paster et al., 2001). The most conventional methods used for analysing bacterial communities do not take the unculturable proportion into consideration (Amann et al., 1995). Thus most of the new technologies being developed for the study of microbial ecology are based on the molecular phylogeny of rRNA, particularly the 16S rRNA gene (Rappe and Giovannoni, 2003). Earlier workers (Kroes et al., 1999) have demonstrated that a greater microbial diversity could be ascertained by amplifying rDNA directly from subgingival plaque compared to that cultivated from the same specimen. Currently, the best model for exploring microbial diversity involves PCR-cloning of the 16S rRNA gene (Spratt et al., 1999; Becker et al., 2002). Cloning and sequencing of multiple plaque samples is an extremely laborious and expensive task to perform thoroughly due to the huge diversity involved (Paster et al., 2001). For this purpose, genetic fingerprinting techniques may be better suited.

1.7.1 Fingerprinting techniques

There are limited and less developed fingerprinting techniques that analyse the functional diversity of complex microbial communities as opposed to structural diversity. An example of a fingerprint technique that measures functional diversity is community level physiological profiling (CLPP). CLPP measures substrate oxidation profiles through the use of Biolog microtitre plates (Trust Way, Hayward, USA), which allows for intensive spatial and temporal analysis of microbial communities. Other fingerprint techniques, measuring structural diversity, depend on molecular techniques. Examples of these techniques include denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP) and terminal restriction analysis (t-RFLP) of PCR-amplified 16S rRNA genes, all of which provide rapid and cost effective means of analysing bacterial communities (Kerkhof et al., 2000; Fujimoto et al., 2003; Zijnge et al., 2003; Groessner-Schreiber et al., 2004; Junca and Pieper, 2004). *In situ* analysis of microbes has been further facilitated by combining advanced microscopy with a range of molecular approaches such as fluorescently labelled probes, reporter genes or *in situ* PCR (Pernthaler et al., 1998; Korber et al., 1999; Ito et al., 2004). The objective of such studies is to gain some understanding of the population structure, compartmentalisation of bacterial communities, specific gene expression and transfer without disturbing the complex interactions within, for example, dental biofilms.

Culture-independent fingerprinting techniques that rely on PCR tend to provide a pattern or profile of the community diversity based upon the physical separation of unique nucleic acid species (Muyzer, 1999). These methods are rapid and cost effective in comparison to PCR-cloning, but more importantly they allow for the simultaneous analysis of multiple samples. This makes it possible to compare the richness/diversity of microbial communities from different habitats. As an example, the microbial diversity of dental plaque sampled from several patients with and without gingivitis can be simultaneously compared by using these techniques in a cross-sectional study. This technique can also be used in longitudinal studies to

observe for changes in the bacterial community structure within an individual over a period of time.

There are various genetic fingerprinting techniques and these can be divided into 'direct methods', where extracted nucleic acids are directly analysed, such as low-molecular-weight (LMW) RNA profiling and 'indirect methods' such as DGGE, temperature gradient gel electrophoresis (TGGE), SSCP, randomly amplified polymorphic DNA (RAPD) or DNA amplification fingerprints (DAF), amplified ribosomal DNA restriction analysis (ARDRA), bisbenzimide-polyethyleneglycol (Bp-PEG electrophoresis), RFLP, tRFLP and fluorescent RFLP (Flu-RFLP) (Muyzer, 1999). The genetic fingerprinting analysis used in this study has been limited to the use of DGGE and hence a focus on this technique and applications will follow.

1.7.2 Community level physiological profiling

This technique is based upon substrate utilisation and has been used reproducibly to investigate spatial and temporal changes in microbial communities in a broad range of environments such as soil, water, plant rhizosphere and phyllosphere and activated sludge (Garland and Mills, 1991; Garland, 1997; Verschuere et al., 1997; Konopka et al., 1998; Kerkhof et al., 2000; Roling et al., 2000; Flores-Vargas and O'Hara, 2006).

CLPP of whole microbial communities involves the use of commercially available 96 well microtitre plates, containing 95 different carbon substrates and a redox dye (tetrazolium violet). This type of assay measures the oxidative metabolism of these substrates to generate patterns of substrate utilisation. The dye is used to colorimetrically detect the increased respiration that occurs in a cell when it is oxidizing the carbon source. In the presence of a carbon substrate that the cell can oxidise, respiration is increased, which causes an irreversible reduction of the colourless dye to a purple formazan. The development of a purple colouration in a well represents positive utilisation of a specific carbon source (Garland, 1997). If

the cell cannot oxidise the carbon substrate, no respiratory burst occurs and no colour change is observed. The resulting 95 test colour patterns (a "metabolic fingerprint") provide high-resolution identification at species and subspecies levels. Distinctive carbon substrate utilisation patterns have been reported for bacterial populations in soil (Bassio and Scow, 1995). Such patterns might lead to the recognition of a metabolic profile associated with a particular environment or indeed a disease state such as gingivitis or periodontitis.

1.7.3 Denaturing gradient gel electrophoresis

DGGE is one of a group of related methods for mutation detection and screening of DNA fragments. In this technique, double stranded DNA (dsDNA) molecules of the same length but differing in base-pair sequence can be partially separated as they migrate down a polyacrylamide gel containing a linearly increasing gradient of denaturants (Muyzer et al., 1996). Melting of the DNA duplex is influenced by two factors which are (i) the hydrogen bonds between complementary base pairs (GC-rich regions melt at a higher temperature than regions that are AT-rich) and (ii) the attraction between neighbouring bases of the same strand or 'stacking.' Consequently, a DNA molecule may have several melting domains with characteristic melting temperatures (Tm). As concentrations of denaturants increase, domains in the DNA dissociate according to their Tm. The DNA fragments continue to migrate across the denaturing gradient until a transition of helical to partially melted molecules occur, and migration of the molecule will halt (Muyzer et al., 1996) (figure 1.26). Complete strand separation is prevented by the presence of a high melting domain which is artificially created by attaching a 40 bp GC-rich clamp to the 5' end of one of the PCR primers prior to PCR-amplification (Muyzer et al., 1996).

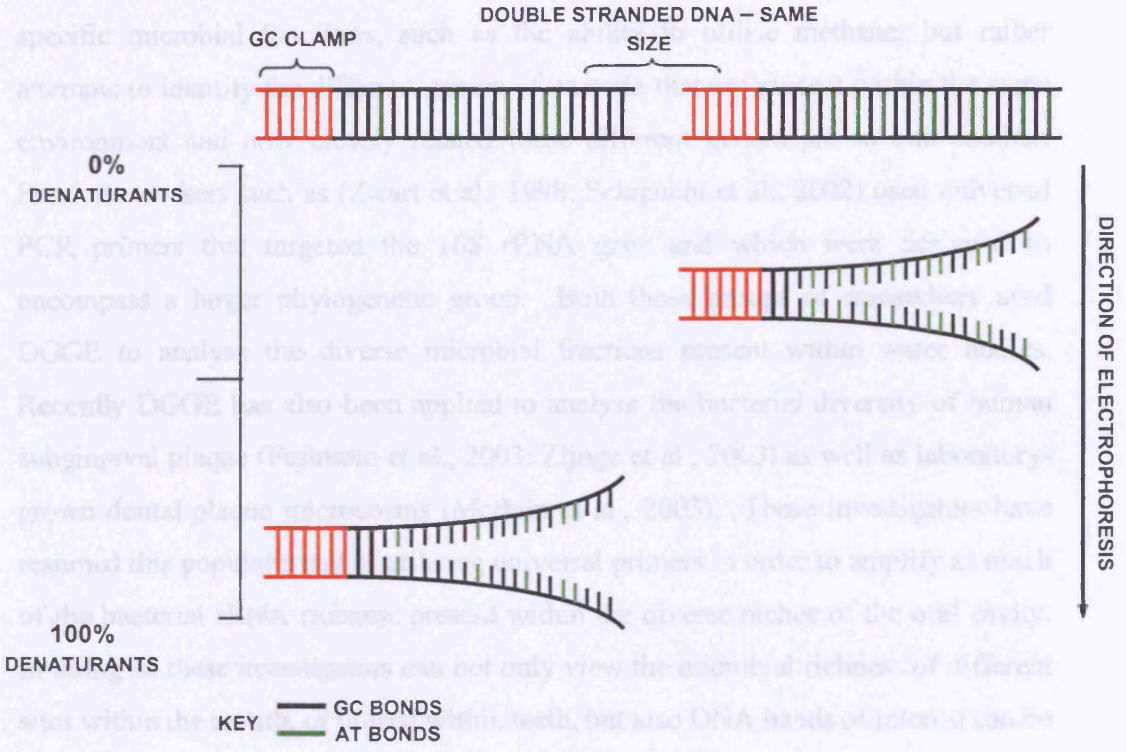


Figure 1.26: Schematic of electrophoretic migration of DGGE fragments. Schematic of DGGE demonstrating the difference in electrophoretic mobility of two DNA fragments of equal size as a result of differences in their base-pair sequence

DGGE has been applied in environmental microbiology (Teske et al., 1996; Boon et al., 2000; Ebie et al., 2002), food microbiology (ben Omar and Ampe, 2000; Ampe et al., 2001) and in the analysis of microbial communities in the human body (Walter et al., 2000; Walter et al., 2001; Favier et al., 2002; Donskey et al., 2003). In general, most of these studies focus on the analysis of the microbial ecology of diverse environments. Some workers use specific rRNA PCR primers in order to selectively amplify the DNA of specific organisms of interest. As an example, previous workers (Bodelier et al., 2005) successfully used DGGE to analyse the diverse methanotrophic fractions of an environment by using 16S-rRNA primers which specifically targeted methane-consuming bacteria. The bacteria identified belonged to different genera but possessed the same methane utilising function. Other efforts also reside on the determination of phylogenetic relationships within

microbial communities. These studies are not biased towards specific genera, or specific microbial functions, such as the ability to utilise methane, but rather attempts to identify the different genera of bacteria that are present within the same environment and how closely related these different genera are to one another. Previous workers such as (Zwart et al., 1998; Sekiguchi et al., 2002) used universal PCR primers that targeted the 16S rRNA gene and which were designed to encompass a larger phylogenetic group. Both these groups of researchers used DGGE to analyse the diverse microbial fractions present within water bodies. Recently DGGE has also been applied to analyse the bacterial diversity of human subgingival plaque (Fujimoto et al., 2003; Zijnge et al., 2003) as well as laboratory-grown dental plaque microcosms (McBain et al., 2003). These investigators have resumed this popular trend of utilising universal primers in order to amplify as much of the bacterial rRNA richness present within the diverse niches of the oral cavity. In doing so these investigators can not only view the microbial richness of different sites within the mouth, or indeed within teeth, but also DNA bands of interest can be excised and sequenced in an attempt to identify genera suspected to be associated with periodontal disease. This technique is not hampered by the 'yet to be cultured' fractions of the oral microbiota and hence, unlike culture-dependent techniques, DGGE might in the future elucidate new genera involved in oral diseases. To date DGGE has not been applied to analyse the microbiota of children with and without gingivitis.

1.8 Aims

(i) To measure the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* present in gingival crevicular plaque sampled from the lower left or right first permanent molar teeth of pre-pubertal children with and without gingivitis.

(ii) To analyse the functional diversity and structural diversity of these dental plaque samples by using CLPP and DGGE

(iii) To determine whether there is a specific metabolic fingerprint or community structure that influences the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in children with and without gingivitis.

(iv) To determine whether there is a specific metabolic fingerprint or community structure that distinguishes the subjects with gingivitis from the subjects without gingivitis.

1.9 Overview

Periodontal disease refers to both gingivitis and periodontitis and periodontitis is extremely rare in healthy children. Gingivitis is widespread among children of all ages, although the clinical manifestations are more aggressive in children and adults with systemic, congenital or genetic disorders. In this study, supragingival plaque from pre-pubertal children with and without gingivitis was sampled. None of the subjects had taken antibiotics or other medication in the preceding 3 months to sampling. Due to the improbability that children in the age group sampled (5-9 years) would smoke or take social drugs, it was assumed that gingivitis was solely due to a result of plaque accumulation. A culture-independent analysis of dental plaque from subjects with and without gingivitis, for the 3 periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* would provide information on the prevalence of these bacteria. Fingerprint analysis using CLPP and DGGE would provide information in the functional and structural diversity of the dental plaque between cohorts. DGGE might identify differences in the bacterial community structure of dental plaque from subjects with and without gingivitis. Furthermore, DGGE fingerprint analysis might also help to determine whether the presence of specific taxa may influence the prevalence of the 3 periodontal pathogens of interest. The aim of this work was to gain a deeper understanding of the microbial diversity of plaque in subjects with and without gingivitis. This knowledge can help further understand the microbial factors that might propagate, or help in the progression of gingivitis to periodontitis.

Chapter 2: Subjects, general materials and methods

2.0 Materials and methods

The subject samples and methods outlined in the following chapter were used throughout the project. Specific methods used are outlined at the beginning of the relevant chapters.

2.1 Subjects

Ethical approval (JREC: 00/E031) was obtained from the Eastman Dental Hospital NHS Trust, London, UK. Each parent was given an information sheet to read and asked for written consent. Each child was asked for verbal consent. Healthy children aged between 5 and 9 years attending the Department of Paediatric Dentistry and the School of Dental Therapy at the Eastman Dental Hospital were recruited. Children with chronic medical disorders, or who had been treated with antibiotics within the preceding 3 months were excluded. Two groups of children were recruited. Group 1 comprised children with discernible plaque and no gingivitis associated with either the lower left or lower right first permanent molar tooth. Group 2 comprised children with discernible plaque and gingivitis associated with either the lower left or lower right first permanent molar tooth. Plaque and gingivitis scores were recorded for each child using a modification of O'Leary (Franco et al., 1996). Four gingivally related quadrisections of each tooth (mesiobuccal, distobuccal, mesiolingual, distolingual) were visually examined to give a score of 0 for no discernable plaque and 1 for discernable bacterial dental plaque deposits. Similarly, each tooth quadrisection associated with no gingival inflammation was given a score of 0 and a score of 1 for gingival inflammation. When added together these numbers gave the gingivitis score. The plaque score and gingivitis score may be divided by the total number of quadrisections in the patient's mouth to provide the plaque index and the gingivitis index. This method is believed to provide a better estimate of oral bacterial loading than other methods since it uses data from every tooth surface adjacent to the gingival margins (Franco et al., 1996).

Dental plaque was sampled from the buccal and lingual gingival crevice of either the lower left or lower right first permanent molar tooth using a sterile toothpick.

Where both lower permanent first molars were erupted either the right or left tooth was randomly selected. The wooden stick was immediately placed in a sterile container with 1 ml of Reduced Transport Fluid (RTF; Syed and Loesche, 1972) and 5 glass beads. The plaque samples were dispersed in the RTF by vortexing for 10 seconds and whole genomic DNA was immediately extracted using the Puregene™ DNA isolation Kit for yeast and Gram-positive bacteria (Gentra Systems, Minneapolis, USA). The genomic DNA was then stored at -80 °C.

2.2 Sources of media, enzymes and reagents

Unless otherwise specified, all media was obtained from Oxoid (Basingstoke, UK), Becton Dickinson (Oxford, UK) and Lab M (Bury, UK). Most chemicals and all antibiotics were obtained from Sigma (Poole, UK). DNA polymerase was obtained from Bioline (London, UK) and dNTPs from Promega (Southampton, UK).

2.3 Bacterial strains

The different bacteria used in this study are summarised in table 2.1. The bacterial strains obtained were either from the National Collection of Type Cultures (NCTC, Public Health Laboratory Services [PHLS]; Colindale, UK), the American Type Culture Collection (ATCC; Manassas, USA) or wild strains identified by comparative 16S rRNA gene sequencing.

2.3.1 Growth of bacterial strains

All the bacterial strains were grown at a temperature of 37°C. Unless otherwise specified, all solid media used was supplemented with 5% defibrinated horse blood (E & O laboratories, Bonnybridge, UK). All bacteria, unless specified, were grown in an anaerobic chamber (MACS 1000, Don Whitley Scientific Ltd, Shipley, UK) with an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Several growth media required supplements as outlined in table 2.1. Broth cultures were supplemented with the same ingredients as those used in solid medium with the exception of the 5% defibrinated horse blood. All anaerobic bacteria were grown in Fastidious Anaerobic Broth (FAB; Lab M) whilst aerobic bacteria were

grown in Todd Hewitt (TH) broth (Oxoid). Subsequent to growth, the bacterial strains were Gram stained and analysed microscopically to check for purity.

2.3.2 Storage of bacterial strains

Several colonies of a given strain were transferred from agar plates into sterile tubes containing 1 ml sterile Brain Heart Infusion (BHI) broth + 30% sterile glycerol. The mixture was vortexed and then frozen at -80°C (Sambrook et al., 1989).

BACTERIA	SOURCE	GROWTH CONDITIONS	REFERENCES
Streptococcus oralis	NCTC 11427^T	Grown on Brain Heart Infusion Agar (BHI [Becton Dickinson]). Anaerobic incubation for 24 hrs.	MI
Streptococcus sanguinis	NCTC 7863^T	Grown on BHI. Anaerobic incubation for 24 hrs.	MI
Streptococcus mutans	NCTC 10449^T	Grown on BHI. Anaerobic incubation for 24 hrs.	MI
Actinomyces naeslundii	Wild type	Grown on BHI. Anaerobic incubation for 24 hrs.	MI
Neisseria sicca	Wild type	Grown on BHI. Aerobic incubation for 24 hrs.	MI
Fusobacterium nucleatum	NCTC 10562^T	Grown on Fastidious Anaerobic Agar (FAA [Lab M]). Anaerobic incubation for up to 3 days.	MI
Tanerella forsythensis	ATCC 43037^T	Grown on Tryptone Soy Agar (TSA [Oxoid]) + 4 g l^{-1} yeast extract + 10 mg l^{-1} N-acetylmuramic acid (NAM, [Sigma]). Anaerobic incubation for up to 7 days.	(Takemoto et al. 1997)
Actinobacillus actinomycetemcomitans	NCTC 9710^T	Grown on TSA + 6 g l^{-1} of yeast extract (Oxoid). After autoclaving the medium was further supplemented with glucose (0.15%) + $NaHCO_3$ (0.4%). Anaerobic incubation for up to 7 days.	(Sreenivasan et al. 1993a)
Porphyromonas gingivalis	NCTC 11834^T	Grown on FAA + 10 μg ml^{-1} haemin + 1μg ml^{-1} vitamin K_1. Anaerobic incubation for up to 7 days.	(Hunt et al. 1986)
Capnocytophaga ochracea	ATCC 27872^T	Grown on FAA. Anaerobic incubation for up to 3 days.	MI
Prevotella intermedia	Wild type	Grown on FAA. Anaerobic incubation for up to 3 days.	MI
Eikenella corrodens	Wild type	Grown on FAA. Anaerobic incubation for up to 3 days.	MI
Veillonella atypica	Wild type	Grown on FAA. Anaerobic incubation for up to 3 days.	MI
Escherichia coli	TOP10 (Invitrogen)	Grown on Luria Broth Agar (LB) (NaCl 10 gl^{-1}, tryptone [Oxoid] 10 g l^{-1}, yeast extract 5 g^{-1}, Technical Agar 20 g^{-1}[Oxoid], ampicillin 100 μg ml^{-1} and kanamycin 50 μg ml^{-1})	MI Invitrogen

Table 2.1: Bacteria and associated growth media used in this study. MI = Manufacturer's instructions

2.4 Genomic DNA extraction from reference strains and plaque samples

The genomic DNA from the dental plaque samples and the reference bacterial strains were extracted individually by using the Puregene™ DNA isolation kit for yeast and Gram-positive bacteria (Gentra Systems, Minneapolis, USA). The reference strains were grown in broth culture prior to the DNA extraction, whereas the DNA from the plaque samples was extracted directly from the RTF suspension. The bacterial reference strains were grown in the appropriate broth medium containing the relevant supplements where necessary. The plaque samples were vortexed into suspension in the RTF, a process facilitated by the presence of glass beads. A 1 ml volume of cells from either bacterial cultures or plaque samples was pelleted by centrifugation at 16 RCF in an Eppendorf 5804R bench top micro-centrifuge (Cambridge, UK) for 1 minute. This was followed by re-suspending the pellet in 600 µl of Cell Suspension Solution (Puregene™ DNA isolation kit) to which 3 µl of lytic enzyme was added. The sample tubes were then inverted 25 times and incubated for 30 minutes at 37°C with occasional inverting. After incubation, the cells were pelleted by centrifugation at 16 RCF for 1 minute and the supernatant was discarded. The pellets were resuspended in 600 µl of Cell Lysis Solution (PuregeneTM DNA isolation kit) and incubated at 80°C for 5 minutes in order to complete the cell lysis. This was followed by the addition of 3 µl of RNase to the cell lysate, inverting 25 times to ensure thorough mixing and then incubating at 37°C for 15 minutes. After incubation, the samples were cooled to room temperature and 200 µl of Protein Precipitation Solution (Puregene™ DNA isolation kit) was added to the lysate. Thorough mixing was then ensured by vortexing at high speed for 20 seconds. Following this, the protein was pelleted by centrifugation at 16 RCF for 3 minutes and the supernatant was poured into a new microcentrifuge tube containing 600 µl of 100% isopropanol. This was mixed by inverting 50 times, centrifuged once more at 16 RCF for 1 minute and the supernatant was discarded. The DNA was then washed by adding 600 µl of 70% ethanol and inverting several times. The samples were then centrifuged at 16 RCF for 1 minute and the supernatant discarded. The DNA pellet was air dried and then re-suspended in 50 µl of DNA Hydration Solution (Puregene ™ DNA isolation kit).

2.5 Polymerase chain reaction (PCR)

The final reaction volume was either 50 or 100 µl. Master mixtures consisted of 10 x PCR buffer, $MgCl_2$, dNTP's and Taq DNA polymerase, the concentrations of which varied for different reactions. The DNA polymerase used in all reactions (unless otherwise specified) was BioTaq (Bioloine, London, UK). A typical PCR consisted of an initial denaturing step of 94 °C for 5 min that ensured the denaturing of double stranded DNA and any secondary structures formed by the primers. This was followed normally by 25-35 cycles of denaturation at 94 °C for 1 min, annealing of primers (temperature varies between PCR) for 1 min and primer extension at 72 °C for 1.5 min. A final extension of 72 °C for 5 min ensued the cycling. The denaturing step was usually carried out at 94-95 °C. The primer annealing step is dependant on the melting temperature (Tm) of the primers. Generally, the annealing temperature (T_A) was 5°C lower than that of the primer with the lowest Tm. The final extension temperature of 72°C was used throughout. All PCRs, unless otherwise specified, were carried out on a Primus thermal cycler (MWG Biotech, Milton Keynes, UK). All primers were obtained from Sigma-Genosys Biotechnologies (Haverhill, UK). The oligonucleotide sequences for the primers commonly used throughout this study are listed in table 2.2.

2.6 Agarose gel electrophoresis

A 5 µl volume of PCR product was mixed with 1 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% sucrose in sterile distilled water) and analysed by agarose (Amresco, Ohio, USA) gel electrophoresis (Sambrook et al., 1989). The gels were made up to the required concentration in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA; Sambrook et al., 1989). All gels contained ethidium bromide at a final concentration of 0.5 µg ml^{-1}. Gels were electrophoresed at between 60-100 V, depending on the size of the PCR product. All gels were visualised and photographed under a UV light transilluminator (AlphaImager San Leandro, USA).

2.7 Purification of PCR products

All PCR products, unless otherwise specified, were cleaned using the QIAquick PCR purification kit (QIAGEN, Crawley, UK).

PRIMER	PRIMER SEQUENCE	REFERENCE OR SOURCE
27 Forward	5'-AGAGTTTGATCMTGGCTCAG-3'	(Lane, 1991)
1492 Reverse	5'-TACGGYTACCTTGTTACGACTT-3'	(Lane, 1991)
357 Forward	5'-CCTACGGGAGGCAGCAG-3'	(Lane, 1991)
518 Reverse	5'-ATTACCGCGGCTGCTGG-3'	(Lane, 1991)
M13 Forward	5'-GTAAAACGACGGCCAG-3'	Invitrogen
M13 Reverse	5'-CAGGAAACAGCTATGAC-3'	Invitrogen
T3	5'-ATTAACCCTCACTAAAGGGA-3'	Invitrogen

Table 2.2: Primers continuously used throughout study. Redundancies are as follows (M = C or A and Y = C or T)

2.8 DGGE profiling – touchdown PCR

The V2-V3 region of the 16S rRNA gene corresponding to position 339-539 of *Escherichia coli* for samples of interest were amplified using the PCR primers 357FGC, which consisted of the 357F primer and a 40 bp GC clamp starting from the 5' end (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCC-GCCCC-3'), and 518R. The amplification reaction mixture (final volume 50 µl) contained 10 x PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM of both primers 357FGC and 518R and 5 U of Taq DNA polymerase. All dNTP's were used at a final concentration of 0.2 mM. The cycling parameters for a touchdown PCR were as follows: After pre-incubation at 94°C for 5 min, 30 cycles were performed at 94°C for 1 min, annealing temperature (T_A) for 1 min and 72°C for 1 min. The T_A decreased step-wise by 1°C every 2 cycles from 65°C in the first cycle to 56°C in the 20th cycle. The T_A for the last 10 cycles was 55°C. Cycling was followed by 5 min incubation at 72°C (Zwart et al., 1998). The expected PCR product length was ca. 235 bp. Unless otherwise specified, all touchdown PCRs were conducted using the above mentioned PCR mixture, primers and cycling parameters.

2.9 Casting parallel DGGE gels

Parallel gels (see figure 2.1 for setup) containing 10% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) were cast using a DCode system (BioRad laboratories Inc., Hercules, CA, USA). The gels contained a linear gradient of the denaturants

urea and formamide, increasing from 40% at the top of the gel to 80% at the bottom (with 100% denaturants corresponding to 7 M urea and 40% [v/v] deionised formamide). PCR products (30 µl) and loading buffer (10 µl) were loaded onto the gels and run at 35 V for 21 hr (735 V hr) at a constant temperature of 60°C in 7 l of 1x TAE buffer (figure 2.2). Gels were stained for 1 hr in 1x TAE containing SYBR Green Nucleic Acid Gel Stain (10^{-4} dilution) (Molecular Probes, PoortGebouw, Netherlands) and photographed under a UV light transilluminator (AlphaImager).

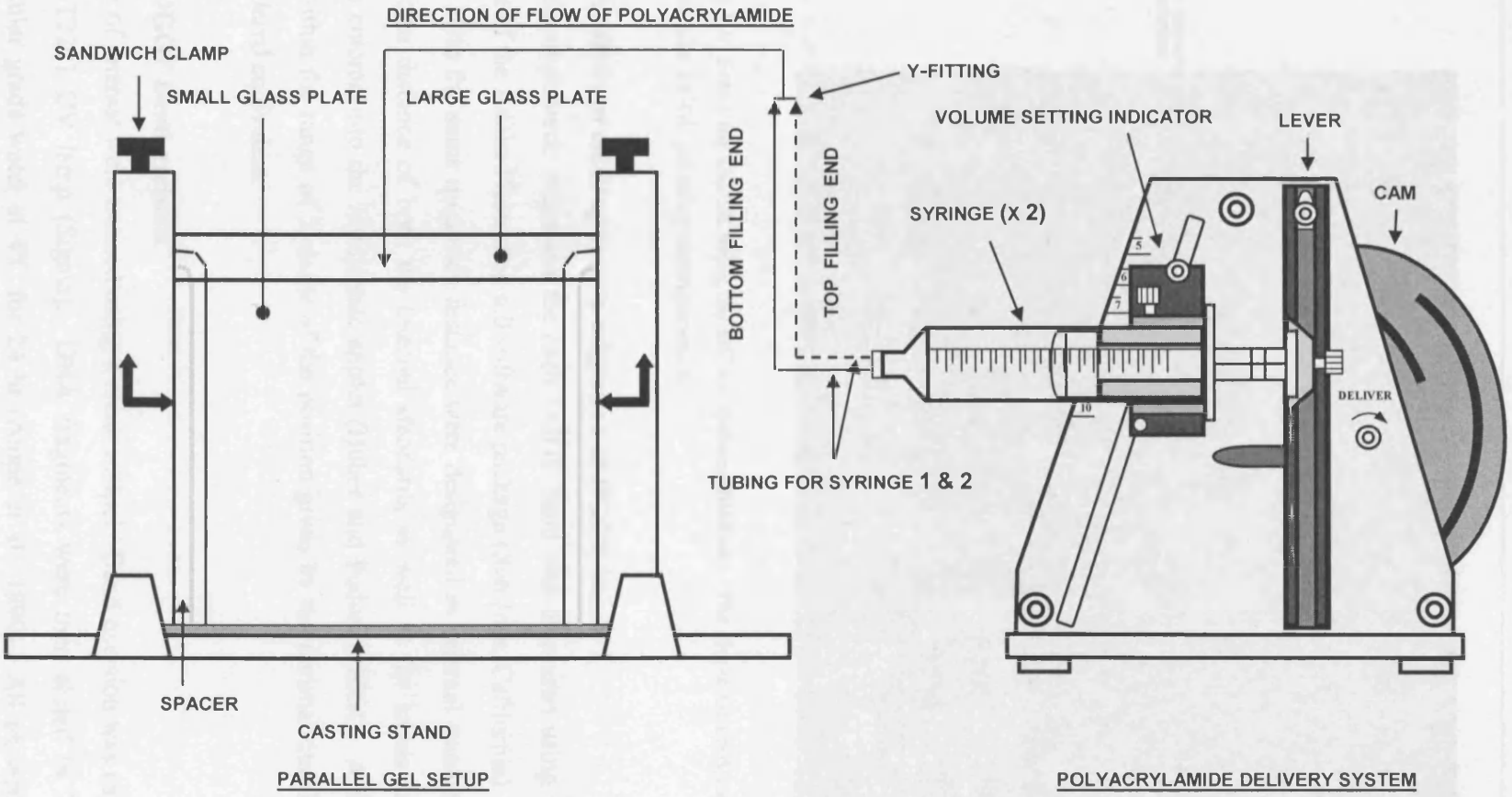


Figure 2.1: System setup for casting parallel DGGE gels

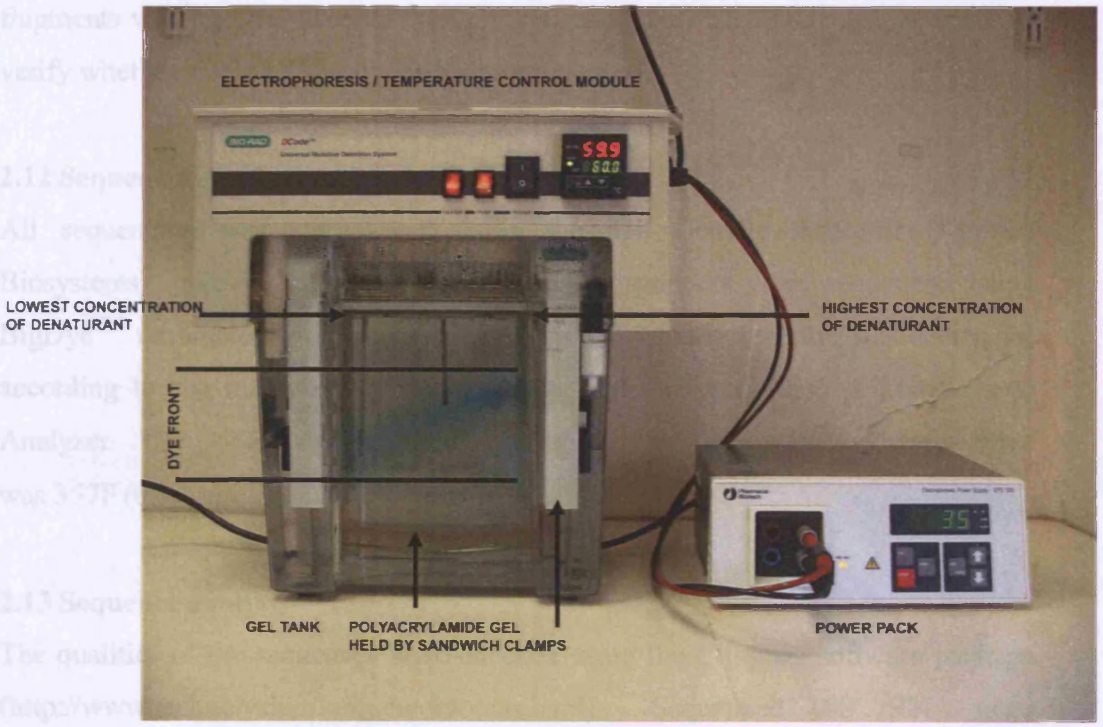


Figure 2.2: Setup for DGGE using the DCode system (BioRad). The above set up demonstrates a perpendicular DGGE gel being electrophoresed

2.10 Analysis of electrophoretic migration of DGGE bands

The electrophoretic migration for each DGGE band was measured using the ruler option of the Adobe Photoshop 6.0 software package (San Jose, California). Pairs of bands with the same migration distance were designated as internal standards. The migration distance of both the internal standards as well as the bands under test where entered into the EquiBands applet (Huber and Peduzzi, 2004). A band that was within the range of 3 pixels of the position given by the optimal matching was considered equivalent.

2.11 DGGE band excision

Bands of interest were excised using a clean scalpel. Band excision was carried out on a T2201 UV lamp (Sigma). DNA fragments were then eluted in 20 µl of molecular grade water at 4°C for 24 hr (Ampe et al., 1999). All excised DGGE

fragments were electrophoresed through a second, identical DGGE gel, in order to verify whether single DGGE bands had been excised.

2.12 Sequencing and sequence determination

All sequencing was carried out using the 310 Genetic Analyzer (Applied Biosystems), unless otherwise specified. PCR products were sequenced using BigDye™ terminator cycle sequencing version 2.0 ready reaction sequencing kit according to the manufacturer's instructions and analysed using a 310 Genetic Analyzer. The primer used in all sequencing reactions, unless otherwise specified was 357F (0.1 μM).

2.13 Sequence analysis

The qualities of the sequences were checked using the Chromas software package (http://www.technelysium.com.au/chromas.html). Sequenced 16S rRNA gene fragments were analysed using the basic local alignment search tool (BLAST) (Altschul et al., 1990).

2.14 Cloning

All cloning of PCR products for sequencing was carried out using the TOPO TA cloning kit (Invitrogen, Paisley, UK). Fresh PCR product (0.5-4 μl, depending on DNA concentration as estimated from the agarose gel) was mixed gently with 1 μl salt solution, 1 μl of the TOPO vector plasmid and sterile water (final volume 5 μl). Incubation of the mixture at room temperature for 5 minutes allowed for ligation of PCR product to the plasmid. A 2 μl volume of the reaction mixtures was then added to individual vials of One Shot chemically competent (TOP10) *E. coli* (Invitrogen), mixed gently and then incubated on ice for 30 minutes. After incubation the cells were heat-shocked for 30 seconds in a 42°C water bath. This was followed by the addition of 250 μl S.O.C. medium (Invitrogen) at room temperature. The cells were then shaken horizontally at 37°C for 1 hour and then 10 and 50 μl volumes were aliquoted onto pre-warmed LB agar. Agar plates were incubated at 37°C for 24 hr.

Chapter 3:
Multiplex PCR for the detection of three major periodontal pathogens

3.1 Introduction

There are few data concerning the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in the plaque of healthy children. The available prevalence data for these pathogens are also contradictory, for example some workers (Watson et al., 1991) have successfully isolated *P. gingivalis* from the plaque of pre-pubertal children, when other workers have not (Zambon, 1996; Kimura et al., 2002). Although periodontal disease is rare in healthy children, it is important to investigate the presence of periodontal pathogens as the permanent teeth start to erupt. The detection of periodontal pathogens before puberty may be helpful in identifying which children need more effective oral health programs in order to minimize the risk of periodontal disease after puberty (Watanabe and Frommel, 1993).

The introduction of molecular techniques, particularly nested PCR has lowered the routine threshold of bacterial detection to as few as 10 cells (Tran and Rudney, 1999). A PCR technique involving sets of primers that target species-specific regions of the 16S rRNA gene of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* has also been developed to demonstrate the presence of these periodontal pathogens in adults (Tran and Rudney, 1999). This study presents an adaptation of this protocol to study the prevalence of these three periodontal pathogens in plaque samples taken from healthy pre-pubertal children aged between 5 and 9 years, with and without gingivitis in a nested multiplex approach.

3.2 Materials and methods

3.2.1 Sample size calculation

Subjects were recruited with the criteria previously described (see section 2.1). The detection of *A. actinomycetemcomitans* is greater in children with gingivitis compared with children with no gingivitis (Okada et al 2000; Müller et al 2001; Kimura et al., 2002). It was expected to detect the organism in 50% of children with no gingivitis. A two group continuity corrected Chi square test with a 0.05 two-sided significance level will have 80% power to detect the difference between a gingivitis group proportion, 0.800 and a non - gingivitis group proportion, 0.500 when the sample size in each group is 45. The plaque and gingivitis scores for both the primary and permanent dentition, for both the male and female subjects of both cohort groups are shown in Table 3.1 and 3.2.

3.2.2 Nested multiplex PCR

Two rounds of PCR were conducted to analyse the detection frequencies of the three periodontal pathogens in the dental plaque sampled from children without gingivitis (group 1 plaque, n = 65) and children with gingivitis (group 2 plaque, n = 53). The first round of PCR was performed using the 27 forward (27F) and 1492 reverse (1492R) primers. The template consisted of whole plaque DNA extractions. The amplification reaction mixture contained 5 mM 10 x PCR buffer, 2.5 mM $MgCl_2$, 0.2 μM of both primers 27F and 1492R and 5U of Taq DNA polymerase. All dNTPs were used at a final concentration of 0.2 mM. The cycling parameters consisted of 30 cycles of: 94°C for 1 minute (except 5 minutes for the first cycle), 54°C for 1 minute and 72°C for 1.5 minutes (except for 5 minutes for the last cycle). Each PCR was carried out with a negative control consisting of sterile de-ionized water in addition to a positive (1 μl) control consisting of DNA extracted from a pure culture of *E. coli* (10 ng μl^{-1}). The expected product length for this PCR was ca. 1500 bp (figure 3.1) and this was compared with a molecular weight DNA marker (PCR Marker, Amresco, Ohio, USA).

The primers chosen for the detection of the 3 putative pathogens targeted specific regions of the 16S rRNA gene. These consisted of 3 forward primers and 1 conserved reverse primer. The expected product lengths were ca. 197 bp for *P. gingivalis*, ca. 360 bp for *A. actinomycetemcomitans* and ca. 745 bp for *T. forsythensis* (figure 3.2). These were compared with a molecular weight DNA marker (1500E bp molecular weight marker, Promega). The nucleotide sequences for the four selected primers were as follows: *P. gingivalis* specific forward primer (PgF), 5'-TGTAGATGACTGATGGTGAAAACC-3'; *A. actinomycetemcomitans* specific forward primer (AaF), 5'-ATTGGGGTTTAGCCCTGGTG-3'; *T. forsythensis*-specific forward primer (BfF), 5'-TACAGGGGAATAAAATGAGAT-ACG-3'; conserved reverse primer (C11R), 5'-ACGTCATCCCCACCTTC-CTC-3' (Tran and Rudney, 1999). Multiplex PCR was carried out as previously described (Tran and Rudney, 1999). The final volume of each PCR reaction mixture was 53.6 µl (comprising 33.6 µl of the master mixture and 20 µl of DNA template). A hot-start step was included in this protocol and AmpliTaq Gold (AB biosystems, Foster City, California, USA) was used. The master mixture comprised 10.3 mM Tris-HCl, 51.3 mM KCl (10 x PCR buffer II), 2.9 mM $MgCl_2$, 0.15 µM primer AaF, 0.74 µM primer BfF, 0.49 µM primer Pgf, 0.47 µM primer C11R and 10U of AmpliTaq gold. The dNTPs included dATP, dCTP and dGTP each at 0.2 mM concentration and 600 mM dUTP. The cycling parameters consisted of 40 cycles of: 95°C for 1 min (except 10 min for the first cycle), 61°C for 1 min and 72°C for 5 min (except for 10 min for the last cycle). Each PCR was carried out with a negative control consisting of sterile de-ionized water as well as a positive control consisting of 1µl genomic DNA extracted from pure cultures of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* all at a final concentration of 10 ng μl^{-1}. Post-PCR analysis was carried out by electrophoreses of the PCR products on a 2.3% agarose gel in Tris-acetate buffer. Randomly selected products from relevant groups were excised from the gel and the DNA purified. The DNA was then sequenced and the identity confirmed using the species specific primers PgF, AaF and BfF at a final concentration of 0.1 µM.

3.2.3 Determination of detection limits

Prior to working with dental plaque, the lowest detection limit for each of the target strains was determined by both one round of PCR using universal primers and by two rounds of PCR using the specific nested primers. This was achieved with 10-fold dilutions of pure cultures of the type strains. Dilutions were based on microscopic counts using a haemocytometer. Cell numbers ranging from 1 to 10^8 cells ml^{-1} for each species were prepared and a whole genomic DNA extraction was carried out. In order to ensure the precipitation of the entire bacterial DNA, carrier DNA (Sambrook et al., 1989) was used. The carrier DNA used was 10 µg of salmon sperm DNA (Sigma). The nested PCR was carried out for the three pathogens individually in the presence of 1 µl of 10 ng μl^{-1} of genomic DNA extracted from *Capnocytophaga ochracea*. The positive controls in both rounds of PCR consisted of 10 ngμl^{-1} of type strain DNA. A further positive control consisting of 10 ngμl^{-1} of *C. ochracea* was also included in the nested PCR. This reaction required the use of 0.2 µM of the universal forward primer (357F) and 0.2 µM of the *Capnocytophaga* genus-specific reverse primer (562R), 5'-CCCTTTAAACCCAATGAT-3'. The expected amplicon size for *C. ochracea* was ca. 240 bp. Two negative controls were included and consisted of 10 ng μl^{-1} of *C. ochracea* and 0.01 µg μl^{-1} of salmon sperm DNA. The positive and negative controls from the first round of PCR were also used as template in the nested multiplex PCR.

3.2.4 Multiplex primer specificity

A nested multiplex PCR using the species-specific primers PgF, AaF, BfF and the conserved reverse primer C11R was performed using genomic DNA extracted from nine oral bacteria other than *P. gingivalis, A. actinomycetemcomitans* and *T. forsythensis*. These oral bacteria comprised *S. oralis*, *S. mutans*, *F. nucleatum*, *C. ochracea*, *A. naeslundii, P. intermedia*, *E. corrodens*, *N. sicca* and *V. atypica*.

3.2.5 Statistical methods

The Chi-square test was used to compare the detection of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in both gingivitis and non-gingivitis

subjects, with $P < 0.05$ defining significance. Associated odds ratios with 95% confidence intervals (95% CI) were calculated. Additionally, logistic regression analysis was performed and odds ratios with 95% CI were calculated from its results using the detection percentages of the 3 pathogens in plaque from subjects with and without gingivitis in order to determine:

(i) If gender and prevalence of the three pathogens were potential risk factors for gingivitis. Gingivitis status was the dependant variable whilst gender and each of the 3 pathogens, which were coded as either present or absent, were the explanatory variables.

(ii) If there were differences in the colonisation of the three pathogens between male and female children. Each of the pathogens was the dependent variable for three logistic regression analyses with gender and the remaining two pathogens as the explanatory variables.

The level of statistical significance was set at $P < 0.05$. The statistical package used was SPSS version 12 (SPSS, Chicago, Illinois, 60606).

3.3 Results

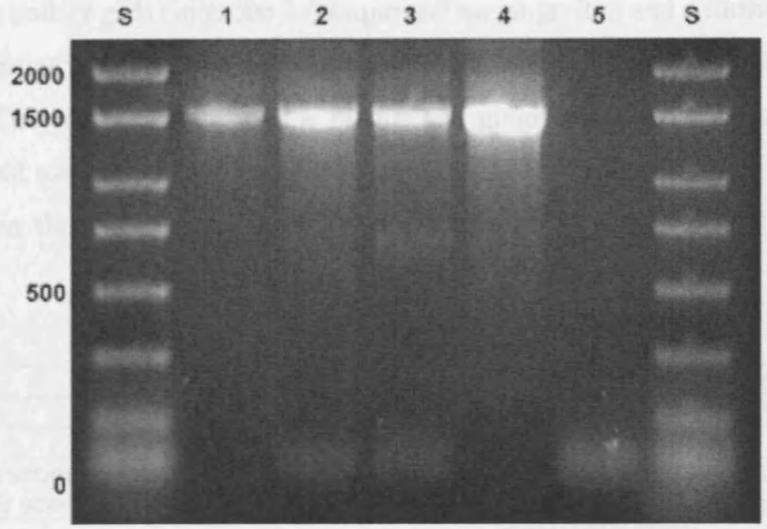


Figure 3.1: PCR products of plaque with the universal primers 27F and 1492R. Electrophoresis of first round PCR products on a 1% agarose gel. S = molecular weight marker; 1 = PCR product for *P. gingivalis*; 2 = PCR product for *A. actinomycetemcomitans*; 3 = PCR product for *T. forsythensis*; 4 = PCR product amplified from a mixture of the 3 pathogens; 5 = negative control consisting of molecular grade water

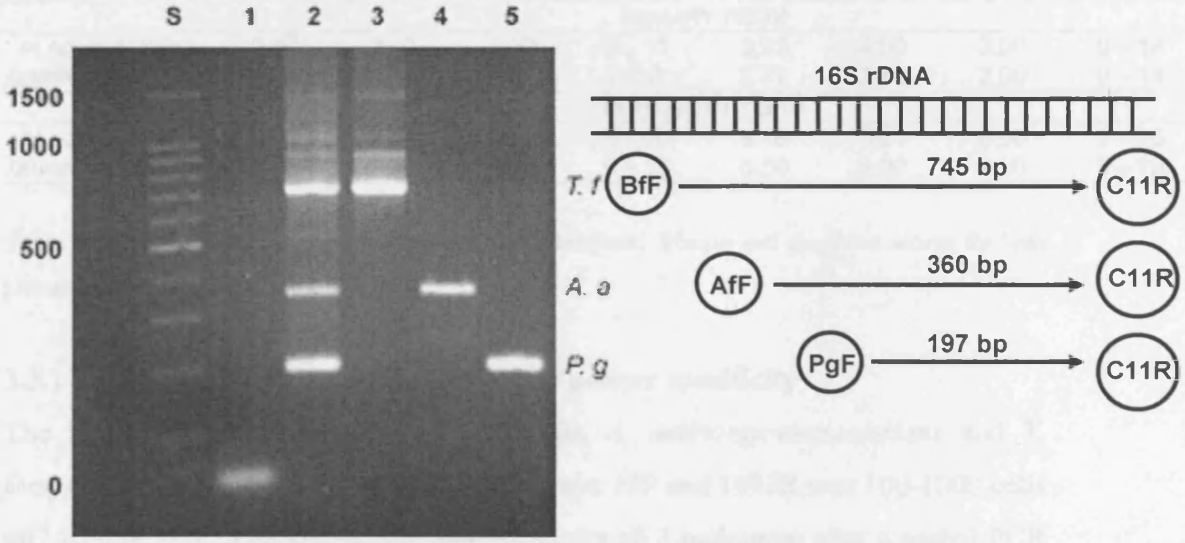


Figure 3.2: Nested multiplex PCR using species-specific and conserved primers: For the simultaneous detection of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. S = molecular weight marker; 1 = negative control consisting of molecular grade water; 2 = PCR product amplified from a mixture of the 3 pathogens; 3 = PCR product from *T. forsythensis*; 4 = PCR product from *A. actinomycetemcomitans*; 5 = PCR product from *P. gingivalis*.

A total of 118 children were recruited, 64 boys and 54 girls. There were 65 children (36 boys and 29 girls) in group 1 (plaque and no gingivitis) and a further 53 (28 boys and 25 girls) in group 2 (plaque and gingivitis). The mean age was 8.3 (SD = 1.1) years. The summary data for the plaque and gingivitis scores for the primary and permanent teeth are shown in tables 3.1 and 3.2. The range of scores is observably greater in the group 2 cohort (plaque and gingivitis) for both male and female subjects.

	GROUP 1 (PLAQUE, NO GINGIVITIS)				GROUP 2 (PLAQUE AND GINGIVITIS)			
	MEAN	SD	MEDIAN	MIN-MAX	MEAN	SD	MEDIAN	MIN-MAX
				PRIMARY TEETH				
PLAQUE SCORE	2.53	2.61	2.00	0 – 8	6.68	8.77	6.00	0 – 45
GINGIVITIS SCORE	0.50	1.183	0.00	0 – 5	3.64	6.52	2.00	0 – 30
				PERMANENT TEETH				
PLAQUE SCORE	3.19	3.62	2.00	0 – 13	8.21	8.80	6.00	0 – 38
GINGIVITIS SCORE	1.78	2.94	0.00	0 – 11	5.25	6.43	3.00	0 – 30

Table 3.1: Plaque and gingivitis scores – Male subjects. Plaque and gingivitis scores for both primary and permanent dentition

	GROUP 1 (PLAQUE, NO GINGIVITIS)				GROUP 2 (PLAQUE AND GINGIVITIS)			
	MEAN	SD	MEDIAN	MIN-MAX	MEAN	SD	MEDIAN	MIN-MAX
				PRIMARY TEETH				
PLAQUE SCORE	3.79	3.49	3.00	0 – 11	3.97	4.00	3.00	0 – 14
GINGIVITIS SCORE	1.24	1.92	0.00	0 – 8	2.72	3.73	2.00	0 – 14
				PERMANENT TEETH				
PLAQUE SCORE	6.40	5.34	6.00	0 – 20	9.16	7.34	8.00	0 – 25
GINGIVITIS SCORE	2.88	3.22	2.00	0 – 13	6.56	5.02	7.00	0 – 19

Table 3.2: Plaque and gingivitis scores – Female subjects. Plaque and gingivitis scores for both primary and permanent dentition

3.3.1 Determination of detection limits and primer specificity

The lowest detection limit for *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* via PCR using the universal primers 27F and 1492R was 100-1000 cells ml^{-1} (figure 3.3). The lowest detection limits for all 3 pathogens after a nested PCR was 10 cells ml^{-1} (figure 3.4). Figures 3.3 and 3.4 demonstrate typical results for detection limits and are representative for only *T. forsythensis*. Results for *P. gingivalis* and *A. actinomycetemcomitans* were similar but with different PCR product size. No *P. gingivalis, A. actinomycetemcomitans* or *T. forsythensis-*

specific amplicons were observed with the negative controls. Specific primers for the 3 target organisms were used to amplify a range of oral bacteria. No cross-reactions were observed.

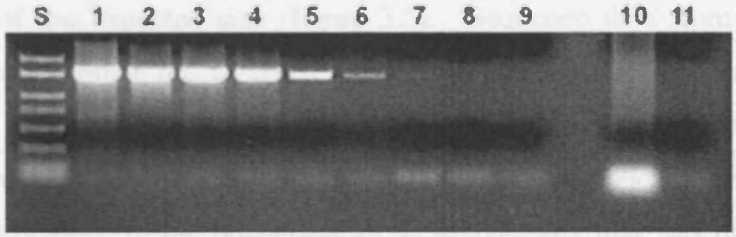


Figure 3.3: Detection limits for the universal primers 27F and 1492R. Lowest detection levels for *T. forsythensis* after a first round of PCR using the universal 16S rRNA oligonucleotide primers 27F and 1492R. S = molecular weight marker; 1 = extracted DNA from 10^8 cells ml $^{-1}$; 2 = extracted DNA from 10^7 cells ml $^{-1}$; Lane 3 = extracted DNA from 10^6 cells ml $^{-1}$; 4 = extracted DNA from 10^5 cells ml $^{-1}$; 5 = extracted DNA from 10^4 cells ml $^{-1}$; 6 = extracted DNA from 10^3 cells ml $^{-1}$; 7 = extracted DNA from 10^2 cells ml $^{-1}$; 8 = extracted DNA from 10^1 cells ml $^{-1}$; 9 = extracted DNA from 1 cell ml $^{-1}$; 10 = Salmon sperm carrier DNA; 11 = negative control consisting of molecular grade water

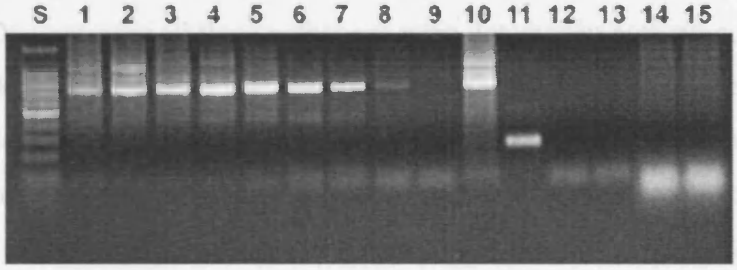


Figure 3.4: Detection limits for the species-specific primer BfF. Lowest detection levels for *T. forsythensis* after a nested PCR using the species-specific primer BfF and the conserved reverse primer C11R. S = molecular weight marker; 1 = PCR product from 10^8 cells ml $^{-1}$; 2 = PCR product from 10^7 cells ml $^{-1}$; 3 = PCR product from 10^6 cells ml^{-1}; 4 = PCR product from 10^5 cells ml^{-1}; 5 = PCR product from 10^4 cells ml^{-1}; 6 = PCR product from 10^3 cells ml^{-1}; 7 = PCR product from 10^2 cells ml^{-1}; 8 = PCR product from 10^1 cells ml^{-1}; 9 = PCR product from 1 cell ml^{-1}; 10 = positive control comprising (10 ngµl^{-1}) DNA amplified form the 16S rRNA genes from the *T. forsythensis* type strain ATCC 43037; 11 = positive control comprising of (10 ngµl $^{-1}$) DNA amplified from the 16S rRNA genes from the *C. ochracea* ATCC 27872 (using the 357F and 562R primers). 12 = negative control comprising (10 ngµl^{-1}) DNA amplified from the 16S rRNA genes from *C. ochracea*; 13 = negative control comprising of molecular grade water used in the first round of PCR; 14 = negative control comprising salmon sperm DNA; 15 = negative control comprising of salmon sperm DNA used in the first round of PCR

3.3.2 Nested multiplex PCR

The periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* were scored as positive or negative on the basis of the presence of a clear band of the expected size (figure 3.5). Sequence data from the excised amplicons representing the assumed taxa were analysed and showed complete agreement. The detection numbers for the three organisms in both health and gingivitis are summarised in table 3.3. Chi-squared statistical tests revealed a significant difference in the prevalence of *T. forsythensis* that was detected more frequently in children with no gingivitis (Chi-squared test statistic = 4.4, degrees of freedom (df) = 1, P = 0.03, 95% CI for difference in detection percentages is 1.6% to 37.0%). There was no statistically significant difference with respect to the prevalence of *P. gingivalis* (Chi-squared test statistic = 0.1, df = 1, P = 0.8, 95% CI for difference in detection percentages is -16.1% to 20.1%) or *A. actinomycetemcomitans* (Chi-squared test statistic = 0.1, df = 1, P = 0.9, 95% CI for difference in detection percentages is -21.0% to 14.8%) in children with and without gingivitis. On occasions all three pathogens were detected in both plaque groups, on other occasions just two of the pathogens were detected and indeed in other occasions just one or none of the pathogens could be detected by multiplex PCR. The different bacterial groupings and their detection in both cohorts have been summarised in table 3.4. Because all categories were mutually exclusive, a Chi-squared test was performed for the data in table 3.4, the results demonstrated no significant relationship between the different bacterial groupings and plaque groups (Chi-squared test statistic = 8.98, df = 7, P = 0.26).

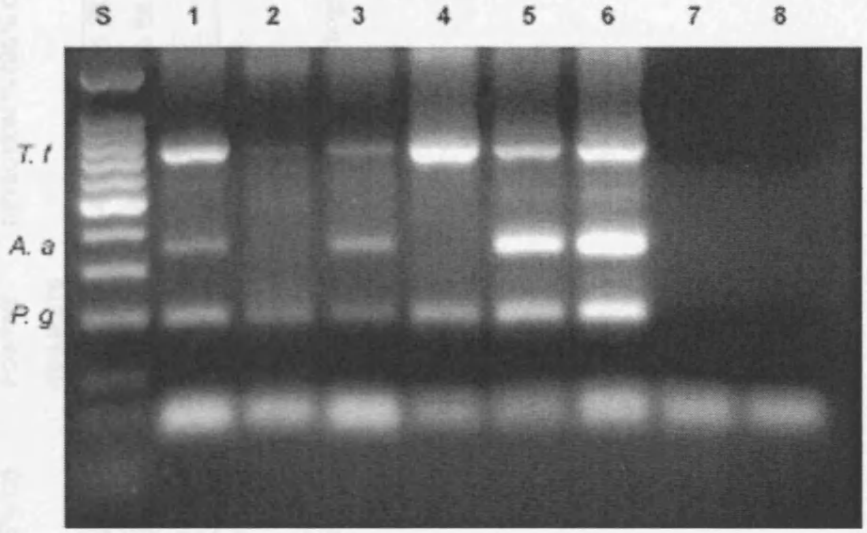


Figure 3.5: Results of a multiplex PCR. Multiple DNA bands observed for the three pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* after multiplex PCR. S = molecular weight marker. The expected amplicon size for the three pathogens are ca. 197 bp, ca. 360 bp and ca. 745 bp and are marked by the symbols Pg, Aa and Bf. Lanes 1-6 comprise of DNA amplified from the 16S rRNA genes present in dental plaque. 1 = amplicons of the expected size for all three pathogens; 2 = an amplicon of the expected size for *P. gingivalis* and a weak amplicon for *T. forsythensis* can also be observed; 3 = amplicons of the expected size for all three pathogens; 4 = amplicons of the expected size for *P. gingivalis* and *A. actinomycetemcomitans*; 5 = amplicons of the expected size for all three pathogens; 6 = positive control comprising of (10 $ng\mu l^{-1}$) DNA amplified from the 16S rRNA genes from the type strains *P. gingivalis* NCTC 11834, *A. actinomycetemcomitans* NCTC 9710 and *T. forsythensis* ATCC 43037; 7 = negative control comprising of molecular grade water; 8 = negative control comprising of molecular grade water used in the first round of PCR

GROUP	SUBJECTS	*P. gingivalis*		*A. actinomycetemcomitans*		*T. forsythensis*	
		POSITIVE SUBJECTS	DETECTION % (95% CI)	POSITIVE SUBJECTS	DETECTION % (95% CI)	POSITIVE SUBJECTS	DETECTION % (95% CI)
1	65	32	49.2% (33.6% to 61.9%)	36	55.4% (42.5% to 67.7%)	42	64.6% (51.8% to 76.1%)
2	53	25	47.2% (33.3% to 61.4%)	31	58.5% (44.1% to 71.9%)	24	45.3% (31.6% to 59.6%)

Table 3.3: Prevalence data for *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Number of subjects in which each of the three pathogens were detected for the two subject groups (group 1 with no gingivitis, group 2 with gingivitis)

COHORTS	DIFFERENT COMBINATION OF BACTERIAL GROUPING DETECTED BY MULTIPLEX PCR							
	Pg / Aa / Tf	Pg / Aa	Pg / Tf	Aa / Tf	Pg	Aa	Tf	None
Group 1	20	4	4	8	4	4	10	11
Group 2	9	7	4	5	5	10	6	8

Table 3.4: Prevalence data for different bacterial groupings of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Number of subjects in which different bacterial groupings of all three periodontal pathogens *P. gingivalis* (Pg), *A. actinomycetemcomitans* (Aa) and *T. forsythensis* (Tf) were detected in both cohorts (group 1 with no gingivitis and group 2 with gingivitis)

3.3.3 Logistic regression analysis

(i) The odds of a subject having gingivitis were 2.3 times greater in the absence of *T. forsythensis* after adjusting for the gender and prevalence of each of the 3 pathogens (P = 0.03, 95% CI for odds ratio [OR] = 1.1 to 4.9). This analysis demonstrated that gingivitis did not appear to be influenced by either gender (P = 0.9, OR = 1.1, 95% CI = 0.5 to 2.2) or the presence of *P. gingivalis* (P = 1.0, OR = 1.0, 95% CI = 0.5 to 2.2) or *A. actinomycetemcomitans* (P = 0.5, OR = 0.8, 95% CI = 0.3 to 1.7).

(ii) There was no statistically significant difference in colonisation among the 3 pathogens in male and female children after adjusting for the other pathogens. In particular, the odds of detecting *P. gingivalis* and *A. actinomycetemcomitans* was greater in females than males [OR = 1.48 (95% CI = 0.68 to 3.21) P = 0.33; OR = 1.30 (95% CI = 0.60 to 2.82) P = 0.51, respectively]. The odds of detecting *T. forsythensis* in females was reduced by 25% compared to those of detecting *T. forsythensis* in males [OR = 0.75 (95% CI = 0.35 to 1.60) P = 0.45].

3.4 Discussion

The purpose of this study was to estimate the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in plaque associated with the lower first permanent molars in prepubertal children. The lower first permanent molars were sampled because these are the first permanent teeth to erupt and periodontal disease does not usually develop before puberty. The use of toothpicks for sampling has been used successfully by other workers (Wennerholm et al., 1995; Tanner et al., 2002a) and is a quick, easy and effective method of collecting both small and large volumes of supragingival plaque.

The species-specific primers used in this study were designed and used by previous authors (Tran and Rudney, 1996, 1999) although in this study the primers are used in a nested PCR for the simultaneous detection of the three pathogens of interest. The replacement of dTTP by dUTP was to allow for the potential use of uracil-DNA glycosylase if amplicon contamination needed eliminating (Longo et al., 1990). In addition to primer specificity analyses, amplicons of the three pathogens from plaque were excised, eluted and sequenced. Sequences obtained were compared with those deposited in public access databases using BLAST, in order to confirm the specificity of the amplicons obtained. The use of genomic DNA belonging to the oral Gram-negative bacterium *C. ochracea* was added to the reaction mixtures for the detection limit analysis so as to mimic 'background' DNA arising from other organisms and host cells which would have been present in the plaque samples. The genomic DNA from *C. ochracea* in the "limit of detection" analysis did not appear to interfere with the PCR. There was a potential risk when preparing genomic DNA extracts for the limit of detection analysis that the DNA from low cell number cultures would remain un-precipitated. The use of carrier DNA in sufficient concentration can be used to encourage the recovery of small amounts of DNA (Sambrook et al., 1989). Using eukaryotic carrier DNA such as salmon sperm encourages the DNA extracted from low cell numbers of bacteria to precipitate effectively.

There were no amplicons specific for *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* observed when using salmon sperm DNA as a second negative control. The nested multiplex PCR detection methodology used in the present study has a limit of detection of ten bacterial cells for all three primers and is similar to those reported by earlier workers (Tran and Rudney, 1999). Nested multiplex PCR can therefore be considered as a more sensitive approach for bacterial detection when compared to other methods such as DNA hybridisation assays where the lowest detection reported has been of the order of 10^2 to 10^3 cells (Chuba et al., 1988). In this present study, a failure to detect a band was interpreted as either the bacteria were absent, or present in numbers lower than 10 cells in the plaque sample. Any weak or faint DNA bands (concentrations under 1 $\mu g\ ml^{-1}$) on gel photographs were regarded as such or indeed as possible DNA carry over from other wells. PCR reactions were repeated for a number of plaque samples to either confirm the presence of weak bands or as a quality control. An example of this can be seen in figure 3.5, lane 2, where a weak band of the expected size for *T. forsythensis* was considered to be negative after repeating the PCR.

The pathogen *T. forsythensis* was observed significantly more frequently in children without gingivitis. Although this is statistically significant, the clinical significance of this finding has yet to be determined. Furthermore, Chi-square tests of the different bacterial groupings demonstrated that there was no significant relationship between the different bacterial groupings and both plaque groups. The significantly higher detection rate of *T. forsythensis* in children without gingivitis was unexpected. This higher prevalence in children without gingivitis can be explained in terms of a possible low stringency of the *T. forsythensis*-specific PCR primer, BfF, for other phylogenetically related organisms. Sequencing of these nested multiplex PCR products demonstrated that the amplified DNA always corresponded to the target organisms *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Nevertheless, there are several taxa which share similar 16S rRNA sequences and hence might not be distinguished accurately by PCR-sequencing. Earlier workers (Paster et al., 2001) have detected a novel *Bacteroides* phylotype, oral clone BU063,

which appears to be significantly associated with oral health (Leys et al., 2002). The available 16S rRNA of this phylotype differs from that of *T. forsythensis* by 9%. If the sequence complementary to the species-specific primer, BfF, is identical for both *T. forsythensis* and BU063, then the prevalence data for *T. forsythensis* will be inaccurate as a result of possible false-positives. *In silico* analysis to verify whether this was indeed the case was not possible because the full 16S rRNA sequence for this phylotype was not available at the time of study. There are other less homologous target regions that can be used to distinguish between both organisms. The intergenic spacer region (ISR) between the 16S and 23S rRNA gene of BU063 and *T. forsythensis* are different in terms of the size and sequence of their non-coding regions (Leys et al., 2002). These two organisms can thus be distinguished by PCR-amplifying their ISRs with 16S forward and 23S reverse primers. This has been successfully demonstrated by previous workers (Leys et al., 2002) who noted that BU063 produces a smaller PCR product (1.48 Kb) than *T. forsythensis* (1.61 Kb).

It is believed that there might be a specific exclusionary mechanism between both species as they are found together less often than would be expected by chance ($P < 0.0001$), with BU063 being more prevalent in healthy sites than *T. forsythensis* (Leys et al., 2002). BU063 might possibly be present in higher numbers in children without gingivitis in comparison to the numbers of *T. forsythensis* present in children with gingivitis. In such a case, the inability to distinguish between both species by using the PCR primer BfF may explain our data. It would be interesting to investigate the prevalence of BU063 and *T. forsythensis* in children with and without gingivitis. It is conceivable that BU063 might be more prevalent and/or in higher numbers in the dental plaque of pre-pubertal children than *T. forsythensis*. Perhaps later changes in the host physiology as a result of puberty, or indeed changes in social habits, might confer a positive bias on the pathogenic *T. forsythensis*. This selective advantage might allow for *T. forsythensis* to out-compete BU063 as the host matures into adulthood. This is an interesting hypothesis because periodontitis is extremely rare in healthy children whilst it is

common in adults. It might be that the changes that occur during puberty could provide the optimal conditions for several pathogenic bacteria to out-compete oral taxa associated with health. The extent to which this happens may determine whether a person develops periodontitis in the future.

There was no significant difference in the prevalence of *P. gingivalis* or *A. actinomycetemcomitans* between the two groups of children. Logistic regression analysis revealed that gender was not a potential risk factor for the development of gingivitis. It also demonstrated that the colonisation of plaque by these three pathogens was not influenced by gender. Although the plaque sampled in this study was from children from a varied ethnic background, correlations between ethnicity and pathogen colonisation were beyond the aims and design of the current study. Further studies specifically addressing ethnicity and/or socioeconomic group need to be carried out.

A lower detection frequency for the periodontal pathogens *P. gingivalis* (8.3%), *A. actinomycetemcomitans* (2.8%) and *T. forsythensis* (2.7%) in the permanent dentition of children aged 7 to 8 years have been previously reported in a study involving cultivation of the organisms (Kamma et al., 2000). Other workers (Frisken et al., 1990) failed to detect any of these three pathogens altogether in young children by culture. In the latter study plaque was sampled from children aged 0 to 2.5 years (age of children in the present study was 5 to 9 years), serially diluted 10 fold and then streaked onto selective media. Other culture studies have demonstrated that *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* belong to a group of oral pathogens which are significant markers for destructive periodontal disease in adult subjects (van Winkelhoff et al., 2002). It was demonstrated that *P. gingivalis* and *T. forsythensis* were the strongest bacterial markers for disease and were infrequently cultured from subjects without periodontal bone loss. Since the samples in all of these studies were serially diluted and then cultured, the detection limit for these taxa would have been very high and target bacteria could have been diluted out. Furthermore, sites exhibiting

periodontal bone loss may have increased numbers of these three pathogens therefore enabling more frequent detection by culture after serial dilutions. Other investigators (Kimura et al., 2002) using the same species-specific primer for *P. gingivalis* as that used in the present study failed to detect this pathogen in any of their subjects. Methods analogous to the present study were used with the exception of the use of a single round of PCR (with a stated detection limit of 100 cells). Therefore it is conceivable that these three pathogens may have been present in plaque and not be detected as a result of them being present in low numbers.

In the present study the plaque was obtained from young children between the ages of 5 and 9. However, there is evidence to suggest that the colonisation of *P. gingivalis, A. actinomycetemcomitans* and *T. forsythensis* can occur from as young as 1.5 years (Tanner et al., 1989). Other investigators (McClellan et al., 1996; Lamell et al., 2000) have detected *P. gingivalis* and *A. actinomycetemcomitans* in the plaque of young children using PCR analysis. The pathogen *P. gingivalis* was detected in 40 to 50% of children ranging from 0 to 2 years of age but the greatest prevalence of 60% was observed in teenagers aged 13 to 14 years (McClellan et al., 1996). On the other hand, *A. actinomycetemcomitans* was detected in 25 to 50% of children ranging from 0 to 3 years of age with the greatest prevalence of 58% detected in children aged between 5 and 9 years (Lamell et al., 2000). The use of PCR techniques for the detection of these three pathogens is a more sensitive method than culturing because DNA from low cell numbers can be amplified and identified.

The detection of the three putative periodontal pathogens in the dental plaque from subjects with and without gingivitis suggest that other factors such as pathogen numbers might be responsible for causing inflammation. This substantiates the principles of the ecological plaque hypothesis (Marsh, 1994) which proposes that organisms associated with disease can be present at healthy sites, but at levels too low to be clinically significant. It is, however, evident that these pathogens are present in periodontally healthy children and that colonisation may occur at a very

young age (Tanner et al., 1989). This could also further support the claim that these organisms may truly be endogenous in the oral cavity. What is still uncertain is whether the early colonisation of these three pathogens in children can be regarded as a risk factor for future development of periodontal disease.

The high prevalence of *T. forsythensis* in children with no gingivitis was unexpected. Previous workers (Okada et al., 2001) have failed to detect this pathogen in 2 to 12 year old children with no gingivitis after a single round of PCR (detection limits not stated). Nonetheless other investigators (Tanner et al., 1989) have detected *T. forsythensis* in the plaque of 18% of very young children (aged 19 to 36 months) using the checkerboard hybridisation assay (Socransky et al., 1994) with a detection limit of $>10^4$ cells. The increased sensitivity for detecting these periodontal pathogens through the use of a nested multiplex PCR may help to explain the higher detection frequency observed in the present study.

The next step in the analysis of how these three periodontal pathogens influence gingival inflammation should investigate the actual numbers of these organisms in dental plaque sampled from both subjects with and without gingivitis. A quantitative assay employing, for example, real-time PCR targeted at these three pathogens in sites with and without gingivitis may demonstrate differences in the actual numbers. Using this technique clinical signs and symptoms could be correlated with actual numbers of each of these pathogens. This current work has demonstrated that these bacteria are indeed present in dental plaque whether associated with gingivitis or not. The subsequent investigations in this thesis have used the same plaque samples to analyse community structures of dental plaque via fingerprint analysis. This type of analysis might help verify if there are specific bacteria or bacterial interactions that are significantly associated with plaque from subjects both with and without gingivitis. More importantly, fingerprint analysis may also demonstrate which bacteria or bacterial associations could encourage the prevalence of these three periodontal pathogens.

3.5 Conclusion

The results of this study have shown that the three pathogens can be detected in the plaque of children both with and without gingivitis and specifically that *T. forsythensis* is more frequently associated with dental plaque found at sites without gingivitis.

Chapter 4:
Community level physiological profiling (CLPP)

4.1 Introduction

The survival and diversity of the indigenous oral microbiota can be primarily attributed to the metabolism of endogenous nutrients provided by the host (Marsh and Martin, 1999). A constant source of nutrients in the mouth is saliva which covers all of the internal surfaces of the oral cavity. This is further supplemented by exogenous substrates provided regularly by the host's diet. Previous workers (Littleton et al., 1967) have demonstrated that subjects which were fed via a stomach tube managed to maintain an oral microflora despite the absence of dietary carbohydrates in the oral cavity. Similarly, saliva is also the only nutrient source believed to support the oral microbiota during periods of no dietary intake such as fasting (Smith and Beighton, 1986) or during the night when the host is asleep. On this basis it could also be expected that GCF will act as another nutrient source during periods of no dietary intake. This is indicative that both saliva and GCF contain sufficient nutrients to sustain oral biofilms (Bowden and Li, 1997; Armitage, 2004a). Moreover, saliva is believed to provide the oral microflora with essential substrates required for growth and development even when dietary carbohydrates are present (Bowden and Li, 1997). It has been documented that bacteria in the mouth can react to varying nutrient concentrations. Previous workers (Li and Bowden, 1994) analysed the effect of excess glucose on *in vitro* biofilm development of *S. mutans, S. sanguinis, S. mitis, A. naeslundii* and *Lactobacillus casei.* They demonstrated that these cells accumulated more rapidly when glucose was present in excess. Moreover, the early lag stages of biofilm development (0-6 hr) observed in models under glucose limitation did not occur. The excess glucose also resulted in a significant increase in biofilm cell numbers when compared to the glucose limited biofilms.

The utilisation of key substrates can also determine the ability of some oral bacteria to adhere to tooth surfaces. It has been documented that *S. mutans* can synthesize glucosyltransferases, which convert sucrose into extracellular glucans thus encouraging adherence to the tooth (Kuramitsu, 1993; Tao and Tanzer, 2002). The adherence of *S. mutans* to the surface of teeth is thus sucrose-dependent. This

bacterium has been implicated as a major pathogen of dental carries (Tanzer et al., 2001) and its cariogenicity appears to be dependent on its ability to adhere to teeth and generate metabolic acids. Thus an excess of substrates such as sucrose may be detrimental to oral health.

The main sources of carbon and nitrogen in the mouth are derived from endogenous proteins and glycoproteins (mucins) present naturally in saliva. Past workers (Palmer, Jr. et al., 2003) have demonstrated that coaggregating early colonisers such as *A. naeslundii* were unable to grow in either a planktonic culture or within a biofilm when using saliva as the sole carbon and nitrogen source. On the other hand, *S. gordonii*, another coaggregating early coloniser, was capable of growing under both conditions. It is believed that bacteria such as *A. naeslundii* would need additional growth factors in order to grow. These growth factors could be obtained by the processing of complex salivary glycoproteins by neighbouring species of oral bacteria (Byers et al., 1999). Other examples of such interactions have been proposed such as (A) the production of lactate by streptococci which is used by *Veillonella* species as a carbon and energy source (Mikx et al., 1972); (B) the production of isobutyrate by *Porphyromonas* species and *Prevotella* species, which is essential for the growth of *Treponema* species (Wyss, 1992); (C) the production of p-aminobenzoate by *S. sanguinis* for which *S. mutans* has an absolute requirement (Carlsson, 1971). Thus, the inability of certain bacteria to survive solely on saliva could be overcome by the development of mutualistic partnerships (Palmer, Jr. et al., 2003). Mutualistic interactions might therefore allow for such bacteria to survive *in vivo*. An analysis of the synergistic and concerted actions of these interacting communities could be achieved by determining their collective substrate utilisation profiles.

The analysis of microbial communities has been restricted by the inability to isolate and culture all of the species present, or even a representative subset. Furthermore, identification of the isolated bacteria is both time consuming and difficult, which limits the number of samples that can be processed. In order to avoid these

difficulties a number of techniques have been developed that do not require the isolation of microorganisms but instead measure genetic, structural or functional properties of the whole community. A rapid community-level cultural approach has been developed (Garland and Mills, 1991) and accepted for community-level physiological profiling (CLPP) (Lehman et al., 1995). This approach has been used extensively in the characterisation of microbial communities (Gorlenko and Kozhevin, 1994; Zak et al., 1994). Carbon substrate utilisation patterns have been used both in environmental and ecological microbiology with respect to the analysis of single strains as well as whole microbial communities (Garland and Mills, 1991; Garland, 1997).

Characteristic patterns of community-level substrate utilisation have been reported for microbial communities in a wide array of ecosystems, but have been confined mainly to soil communities (Heuer and Smalla, 1997; Konopka et al., 1998). The CLPP of microbial communities via carbon substrate utilisation has been used increasingly to investigate spatial and temporal changes in microbial communities with the aim of understanding community dynamics in both basic and applied ecological contexts. This permits the community analysis of fine low biomass microbial populations, such as in dental plaque. Studies of the metabolite production by oral bacteria in dental plaque and the nutritional inter-relationships that exist within such biofilms are important for several reasons: (A) to provide a greater understanding of the formation and development of dental plaques; (B) to provide a greater understanding of the nutritional inter-relationships existing in dental plaques associated with different disease states i.e., gingivitis and periodontitis; (C) because metabolic products of oral bacteria are known to be responsible for certain diseases (Singer and Buckner, 1981), knowledge of their production and utilisation within plaque could lead to the development of means of preventing and/or treating the disease; (D) nutritional inter-relationships among plaque bacteria are responsible, in part, for the development of microbial communities that are able to support the growth of organisms capable of inducing

other oral diseases. A detailed knowledge of such inter-relationships may allow timely intervention and the prevention and/or treatment of such diseases.

The major strengths of a CLPP approach for the analysis of microbial communities are its low manpower requirements and a reliance on metabolic traits that might allow for the characterisation of a change in microbial communities. By demonstrating a reproducible fingerprint of substrates utilised by the microbiota of plaque sampled from subjects with no gingivitis, a "metabolome" for gingival health might be established. Similarly, CLPP could be used to establish a metabolome for gingivitis subjects. This would not be used to clinically establish the presence of gingival inflammation as the clinician could easily verify the presence or absence of gingivitis by conducting a visual examination of the subject's gingivae. A metabolic study of the dental plaque microbiota associated with and without gingivitis might yield valuable information with respect to which substrates are essential for maintaining a degree of gingival health, as well as the substrates that should be avoided to prevent gingival inflammation. This might prove useful in the potential development of pre-biotic products composed of substrates that greatly encourage the prevalence of the oral flora associated with health.

4.2 Materials and methods

The 96 well plates used throughout this study were purchased from Biolog (Trust Way, Hayward, USA).

4.2.1 Biolog profiles for *S. sanguinis*

Several colonies of *S. sanguinis* grown on agar (BHI) were transferred into 20 ml bottles of sterile Todd Hewitt (TH) broth (Oxoid). The broths were incubated in an anaerobic chamber at 37°C for 24 hr. The broth cultures where then centrifuged at 16 RCF for 30 minutes using an Eppendorf 5804R (Cambridge, UK). The supernatant was discarded and the pellet was resuspended in 20 ml of 0.85% sterile phosphate buffered saline (PBS, Sigma). The streptococcal suspensions were diluted to an optical density (OD) of 0.5 at 540 nm (Ultrospec 2000 UV/Visible spectrophotometer; Pharmacia Biotech [Biochrom] Ltd, Cambridge, UK). An aliquot of 150 µl of the suspension was transferred into each well of the GP MicroPlates (Biolog). A Biolog GP MicroPlate has been summarised in figure 4.1. Inoculation of the GP MicroPlates was carried out in triplicate within an anaerobic cabinet in order to maintain fixed environmental conditions between experiments. Care was taken to avoid carry-over of chemicals or splashing from one well to another. Following inoculation, each GP MicroPlate was covered with a lid and incubated aerobically at 37°C for 24 hr. Substrate utilisation was assessed by a colorimetric analysis of the reduction of colourless tetrazolium violet to a purple formazan (MRX II plate reader; Dynex technologies, Ashford, UK). Any absorbance (OD_{590}) values which were 140% or greater than that of the blank A1 was recorded as a positive substrate utilisation (Garland, 1997).

4.2.2 Biolog profiles for low inocula of *S. sanguinis*

The same procedures as those described in section 4.2.1 were carried out except that GP MicroPlates were inoculated with a lowered volume of 20 µl of *S. sanguinis*.

4.2.3 Effect of a gel inoculating medium on Biolog profiles

A gel inoculating medium consisting of NaCl (0.4 %), pluronic polyol F68 (0.03 %), gellan gum (0.02%-0.20%) and sodium thioglycolate at a final concentration of 5 mM was used as a medium for inoculating the *S. sanguinis*. A suspension of *S. sanguinis* (OD_{540} = 1.0) was prepared as described in section 4.2.1. A v/v mixture of streptococcal suspension and gel medium was prepared (final OD_{540} = 0.5). A 20 µl final volume of gel/bacterium suspension was inoculated per well. Inoculation and incubation of the GP MicroPlates was carried out in the same way as described in section 4.2.1. These experiments were carried out in duplicate on GP MicroPlates containing a gellan gum concentration of 0.02% and 0.2%. Each experiment was conducted with a negative control comprising of an extra GP MicroPlate inoculated with only the gel inoculating medium and no bacterium suspension.

4.2.4 Effect of lowered concentrations of indicator on Biolog profiles

This experiment required the use of the Biolog SFP2 MicroPlates which do not contain the redox indicator tetrazolium violet. A gel inoculating medium with a final gellan gum concentration of 0.02% was prepared in the same way as described in section 4.2.3, only that a 0.01% concentration of tetrazolium violet indicator (Sigma) was also added. Bacterial suspensions of *S. sanguinis*, *S. oralis* and *S. mutans* (OD_{540} = 1.0) were prepared. A v/v mixture of streptococcal suspension and gel medium was prepared (final OD_{540} = 0.5) for all three streptococci. A 20 µl final volume of gel/bacterium suspension was inoculated per well of the SFP2 MicroPlates. Inoculation and incubation of the SFP2 MicroPlates was carried out in the same way as described in section 4.2.1, except that readings were measured after 4.5 hrs. These experiments were carried out in triplicate.

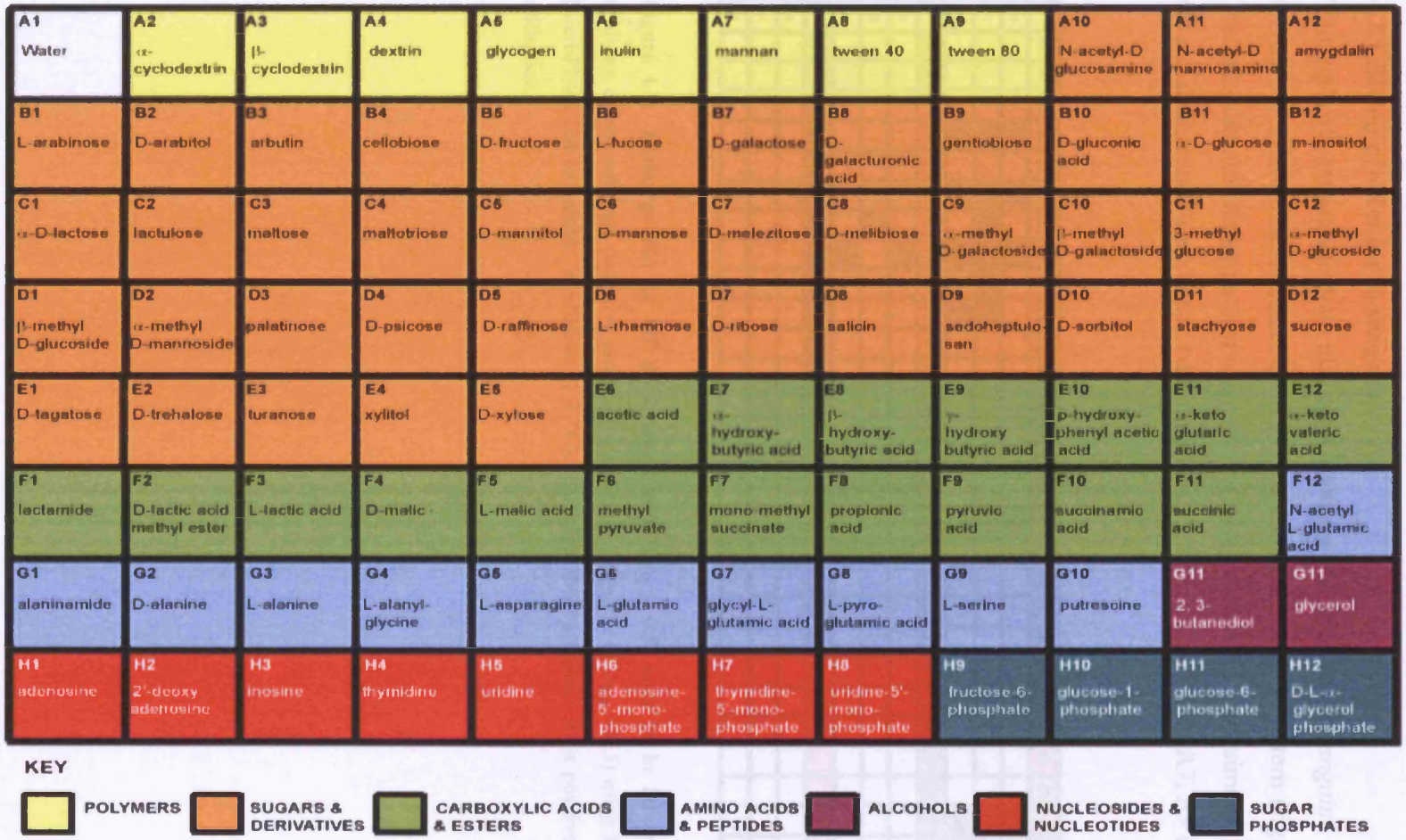


Figure 4.1: List of carbon substrates present in a Biolog GP MicroPlate

4.3 Results

4.3.1 Biolog profiles for *S. sanguinis*

The triplicate carbon substrate utilisation patterns observed for *S. sanguinis* (150 µl, OD_{540} = 0.5) after 24 hrs incubation demonstrated a common pattern (figure 4.2). The substrate utilisation pattern observed for assay n1 and n3 were similar, however several differences were observed for assay n2 (additional positives A7, A9 and D3).

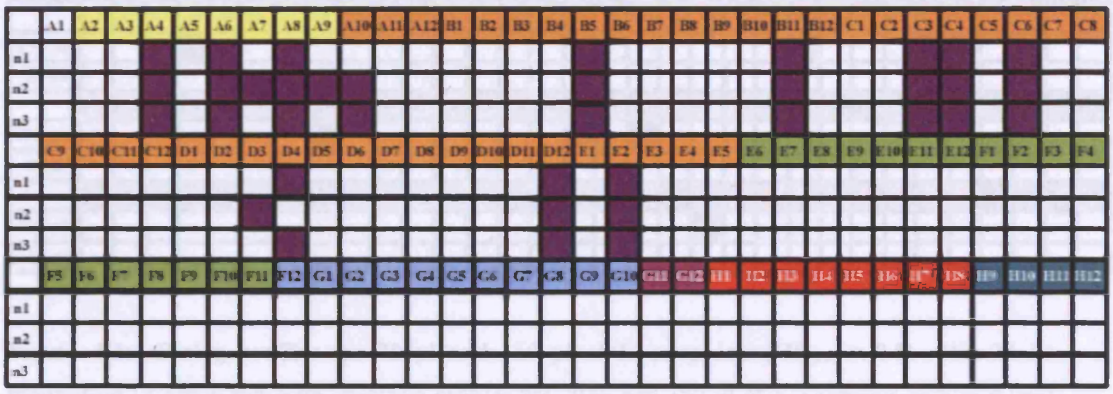


Figure 4.2: Biolog profile for 150 µl *S. sanguinis* (OD_{540} = 0.5) after 24 hr incubation. In triplicate carbon substrate oxidative profiles for 150 µl *S. sanguinis* (OD_{540} = 0.5) using Biolog GP MicroPlates (24 hr aerobic incubation at 37°C). A purple box demonstrates a positive substrate utilisation

4.3.2 Biolog profiles for low inocula of *S. sanguinis*

Lowering the inoculum volume of the *S. sanguinis* suspension (OD_{540} = 0.5) from 150 µl to 20 µl per well resulted in no purple colouration forming as can be observed from figure 4.3. The substrate utilisation profile for 150 µl of *S. sanguinis* observed in figure 4.3 is different to that from figure 4.2. The same strain and concentration of sample was used but on different days, this suggests a lack of reproducibility.

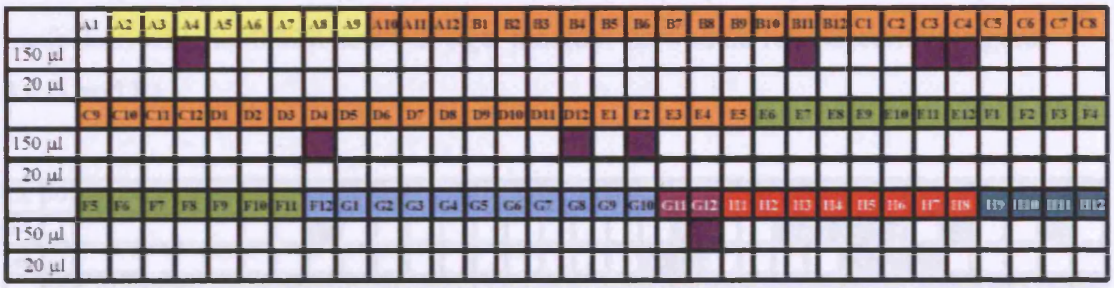


Figure 4.3: Biolog profiles for 20 µl and 150 µl of *S. sanguinis* (OD_{540} = 0.5) after 24 hr incubation. Carbon substrate oxidative profiles for 20 µl and 150 µl of *S. sanguinis* both at a final concentration of OD_{540} = 0.5 using Biolog GP MicroPlates (24 hr aerobic incubation at 37°C). A purple box demonstrates a positive substrate utilisation. No positive utilisation is observed for *S. sanguinis* at the lower aliquot volume of 20 µl

4.3.3 Effect of a gel inoculating medium on Biolog profiles

Positive substrate oxidation was observed for low volume inocula of *S. sanguinis* (20 µl, OD_{540} = 0.5) suspended in a gel medium. The substrate oxidation profiles increased with an increased firmness of the gel, thus a gel medium comprising of 0.02% gellan resulted in less substrates been oxidised than a gel comprising of 0.2% gellan. False positives as a result of the cells metabolising the gel medium were not observed as none of the negative controls (A1 wells) appeared purple. In duplicate carbon substrate utilisation profiles for *S. sanguinis* (20 µl, OD_{540} = 0.5) after 24 hr incubation within a 0.02% and 0.2% gel medium showed no reproducibility (figures 4.4 A and B).

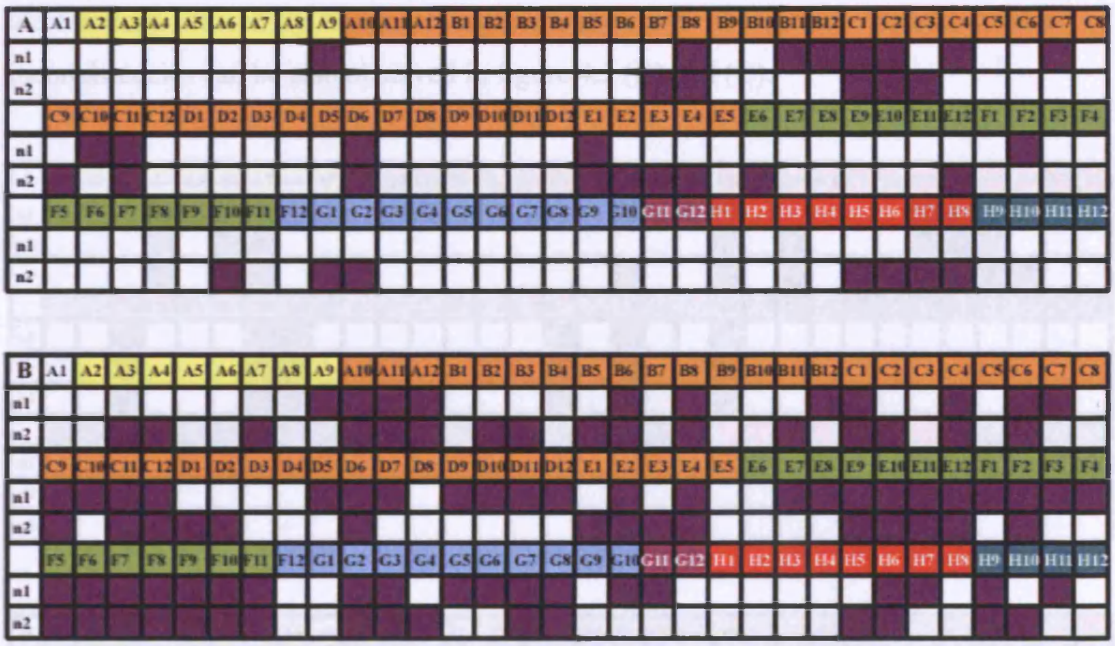
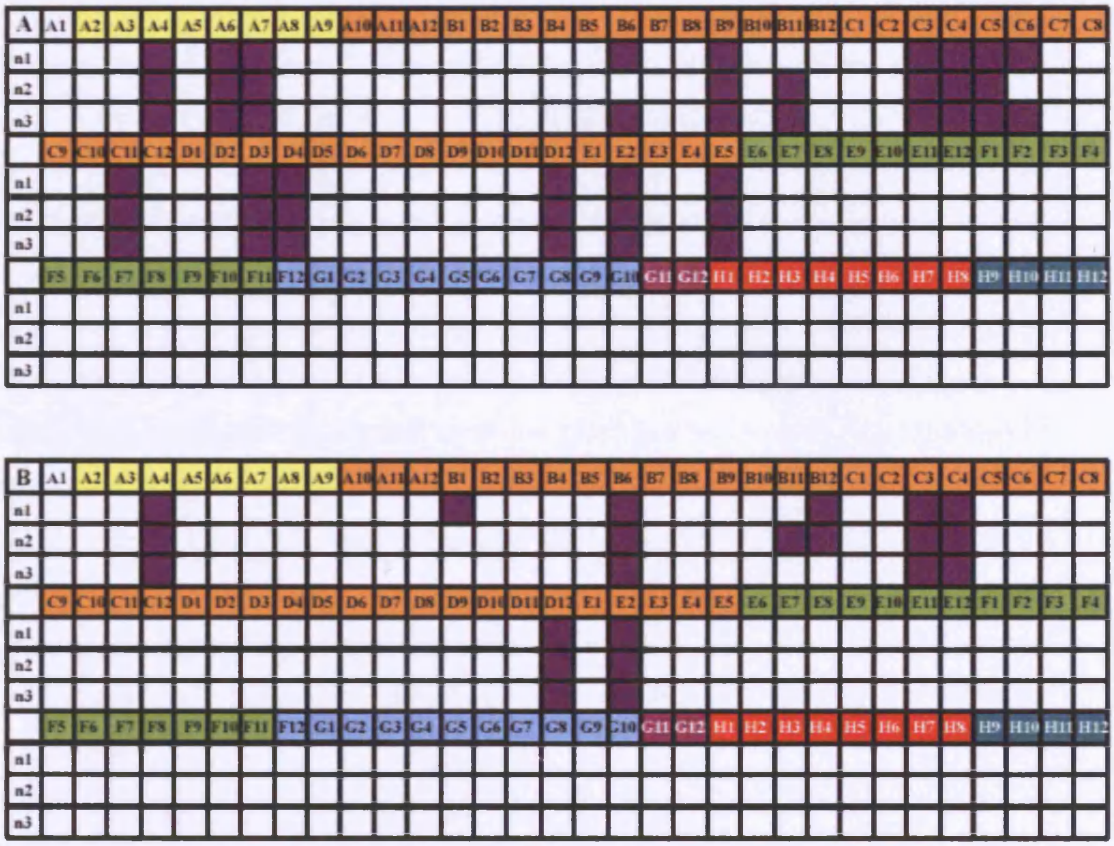


Figure 4.4: In duplicate carbon substrate oxidation profiles for 20 µl *S. sanguinis* (OD_{540} = 0.5) in (A) a 0.02% and **(B)** a 0.2% gel medium using Biolog GP MicroPlates (24 hrs aerobic incubation at 37°C). A purple box demonstrates a positive substrate utilisation

4.3.4 Effect of a lowered concentration of indicator on Biolog profiles

In-triplicate carbon substrate oxidation profiles observed from loading Biolog SFP2 MicroPlates with low volumes of *S. sanguinis*, *S. oralis* and *S. mutans* (20 µl, OD_{540} = 0.5) were more reproducible (figures 4.5 A, B and C, respectively). The streptococci were incubated under the same previous conditions, only that in this case Biolog SFP2 MicroPlates were used as opposed to Biolog GP MicroPlates. The lowered concentration of the tetrazolium violet indicator in a 0.2% gel medium appeared to generate less false positive results. These assays demonstrate that a common fingerprint can be generated for the three different streptococci. Some inconsistencies exist within the triplicate assays e.g. in figure 4.5 (A) substrate B6 and C6 were not utilised by *S. sanguinis* in assay n2. Nevertheless, these substrates were indeed utilised by the same organism in assays n1 and n3. Indeed similar inconsistencies can be also observed in figure 4.5 (B) and (C).



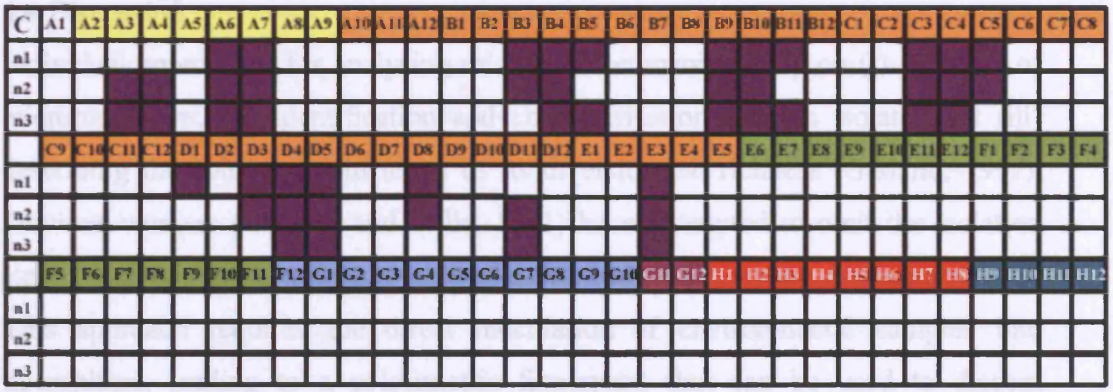


Figure 4.5: Triplicate carbon substrate utilisation pattern for 20 µl of (A) *S. sanguinis*, **(B)** *S. oralis* and **(C)** *S. mutans*, all at (OD_{540} = 0.5) in a 0.2% gel medium (0.01% tetrazolium violet) using Biolog SFP2 MicroPlates (4.5 hr aerobic incubation at 37°C). A purple box demonstrates a positive substrate utilisation

4.4 Discussion

Individual approaches for analysing microbial communities rely on (i) isolation of microorganisms, (ii) identification and characterisation of each isolate, and (iii) describing the community in terms of its diversity and richness (Garland, 1997). Previous workers (Garland and Mills, 1991) have attempted to omit the isolation step of such analyses and have utilised redox technology at the community level. This approach requires the direct inoculation of environmental samples into microplates, leading to a colorimetric fingerprint that can be used to discern differences between microbial communities. A study of the metabolic activity of dental plaque might demonstrate a fingerprint of substrates utilised under conditions of oral health, or indeed conditions of oral disease such as gingivitis.

The main problem when attempting to characterise the CLPP profiles of dental plaque through the use of Biolog MicroPlates was the amount of sample that would be required per plate. For a single assay, a 150 µl aliquot of plaque at a final concentration of OD_{540} = 0.5 was required. This meant that 14,400 µl of plaque would be required per patient, per plate. This would require a relatively large volume of dental plaque from one individual. It was essential to modify the protocols provided by Biolog in an attempt to minimise the volume of aliquot required per well. Prior to working with dental plaque a single species, *S. sanguinis*, was used as a test species to verify whether the lowering of aliquot volumes exerted any changes on the organism's metabolic profile within Biolog GP MicroPlates. A relatively reproducible fingerprint for *S. sanguinis* can be observed when the aliquot volume of 150 µl, as recommended by the manufacturer, is maintained (figure 4.2). There was no colour change observed in the wells when the aliquot volume of *S. sanguinis* was reduced (whilst still maintaining the same optical density) from 150 µl to 20 µl (figure 4.3). This might be as a result of a more confined environment, which might in turn result in excessive toxic conditions that suppress streptococcal metabolism.

To overcome this problem it was suggested that equal volumes of the bacterial suspension and a gel inoculating medium be used (personal communications with Barry Brochner, Biolog's vice president for research and development). A final volume of 20 µl (OD_{540} = 0.5) of this mixture was inoculated per well of each Biolog GP MicroPlate. Duplicate assays revealed no reproducibility between experiments. Furthermore, increasing the gel medium density further increased the number of positive results (figures 4.4 A and B). The fact that the negative control remained colourless in these set of experiments indicated that the positive colour changes within the wells were largely due to the presence of *S. sanguinis*. There is no evidence from these experiments to suggest that such profiles were as a result of the organism's true metabolic preference, but may have occurred as a result of stress (personal communication, Barry Brochner, Biolog). The metabolism of substrates within a confined environment will inevitably lead to an accumulation of metabolic by-products. It stands to reason that this metabolic waste will eventually become toxic to the bacteria within the wells. It would be expected that bacteria within the limited aliquot volume of 20 µl will be subjected to the toxic effects of this metabolic waste at a faster rate than bacteria, of equal concentration (OD_{540} = 0.5), in the larger volume of 150 µl. Thus, the substrate utilisation profiles observed from figures 4.4 A and B might be a measure of toxicity rather than *S. sanguinis* true metabolic preference. In order to minimise these false positives when using a lowered inoculum volume, shorter incubation times where utilised to prevent the accumulation of harmful metabolic waste products, as well as decreasing the concentration of tetrazolium violet indicator. The reduction in concentration of indicator dye should make the assay less sensitive to false positives as a result of stress. The concentration of tetrazolium violet used in this assay would have been approximately 7.5-fold higher as a result of lowering the inoculum volume from 150 µl to 20 µl. The concentration of tetrazolium violet was altered by using Biolog SFP2 MicroPlate. The SFP2 plates are identical to the GP plates only that they do not contain the tetrazolium violet indicator. The tetrazolium violet dye was therefore added to the gel-inoculating medium at a 7.5-fold reduction in concentration. Equal volumes of the bacterial suspension and a 0.2% gel inoculating

medium (with a reduced concentration of the indicator dye) was prepared and 20 µl volumes added to each well of the SFP2 plates. The results obtained by using the SFP2 plates, as observed from figure 4.5 A, demonstrated relatively reproducible substrate utilisation profiles for *S. sanguinis*. More importantly, the same can be said for two other streptococcal species (figure 4.5 B and C) in that in-triplicate substrate oxidation profiles for *S. oralis* and *S. mutans* were also relatively reproducible.

When considering that in triplicate assays for a streptococcal species was carried out using the same bacterial suspension it would be expected that the substrate utilisation profile would be identical in each of the three SFP2 plates. This was not the case as the substrate utilisation profiles for all three streptococci were not 100% reproducible. If on the other hand, in-triplicate assays had been performed on different days, using bacterial suspensions prepared on each consecutive day, minor differences in these profiles could have been explained on the basis that conditions on different days, despite all efforts, would never have been identical. Nevertheless, the results from these experiments have demonstrated that limited reproducibility was achieved with Biolog plates whilst using single species. CLPPs generated from Biolog data were therefore deemed as an impractical way of analysing the metabolic profile of more complex communities, such as dental plaque sampled from a single tooth.

It has also been postulated that only a limited range of microorganisms from a community influence the Biolog substrate utilisation profile (Verschuere et al., 1997). Earlier investigators (Haack et al., 1995) have reported that some culturable strains failed to give a substrate utilisation profile, especially at low densities (below 10^4 CFU ml^{-1}), while at higher densities a clear substrate utilisation profile was observed. Other workers (Ellis et al., 1995) have observed that as little as 10 CFU per well is enough to obtain a positive reaction. Based on such observations, earlier workers (Verschuere et al., 1997) have suggested that the minimal density required to obtain a positive reaction in Biolog plates is strain-dependent. Other investigators

also believe that dilution of the environmental sample should be minimised in order to eliminate bias as much as possible. This would further impede a CLPP analysis of dental plaque from a single site with Biolog plates because not enough sample can be generated without dilution. Other workers (Haack et al., 1995) have also reported that a major effect of sample dilution was a delay in substrate oxidation. Dilution of samples may thus also confer a selective advantage to the fastest growing strains. This may lead to a higher contribution by these strains to the whole community pattern than would be expected from the total organisms within the communities. This has been previously demonstrated by workers who observed that whole community patterns of substrate utilisation were most similar to that of a single species (Verschuere et al., 1997). Thus, such metabolic fingerprints are unlikely to resemble the *in situ* metabolic potential ofa complex microbial community.

It is generally recognised that culture-based techniques are too selective and unrepresentative to be used as an analytical tool to assess microbial community structures (Ward et al., 1990; Wagner et al., 1993). The CLPP approach for analysing microbial community structure does not necessarily identify all of the individual species present in a habitat of interest but can differentiate microbial communities either in terms of structure or function (Lee and Fuhrman, 1990). Consequently, the Biolog system has been used to characterise microbial communities of soil (Gorlenko and Kozhevin, 1994), water (Warren et al., 2004), plant rhizosphere (Kerkhof et al., 2000) and phyllosphere microbial communities (Heuer and Smalla, 1997) as well as many other environmental samples (Konopka et al., 1998). In the case of dental microbiology, CLPP and the Biolog approach for analysing the community structure of single sites is limited at least by sample volume and reproducibility issues. A genotypic approach for the characterisation of dental plaque communities, such as DGGE, will therefore be adopted.

4.5 Conclusion

The use of Biolog plates as a tool for analysing the community structure of dental plaque associated with (i) the prevalence of the three periodontal pathogens of

interest and (ii) the presence or absence of gingivitis, is not a suitable method due to a sample volume constraint as well as an observed limitation on reproducibility. This method might not be the best technique for measuring the functional diversity of a complex microbiota, such as dental plaque. This is because sample dilution has been demonstrated to limit some organism's true metabolic potential. Furthermore, in such confined environments, the metabolic profiles exhibited by CLPP have on occasions been similar to the metabolic profiles of single organisms. Thus, CLPP as measured by BIOLOG plates might be measuring the oxidation profiles of single or several 'fast reproducing' species and hence not reflect the metabolic profile of the whole community. Future work to measure the metabolome of a dental plaque community might include metabolomics.

Chapter 5:
Denaturing gradient gel electrophoresis – A statistical analysis of banding patterns

5.1 Introduction

Denaturing gradient gel electrophoresis (DGGE) is a recently reported fingerprinting technique in which PCR-amplified DNA fragments are separated according to their sequence information (Muyzer and Smalla, 1998). DGGE has been applied in environmental microbiology (Teske et al., 1996; Boon et al., 2000; Ebie et al., 2002), food microbiology (ben Omar and Ampe, 2000; Ampe et al., 2001) and in the analysis of microbial communities in the human body (Walter et al., 2000; Walter et al., 2001; Favier et al., 2002; Donskey et al., 2003). Recently DGGE has also been applied to analyse the bacterial diversity of human subgingival plaque (Fujimoto et al., 2003; Zijnge et al., 2003) as well as laboratory-grown dental plaque microcosms (McBain et al., 2003). Other workers (Torsvik et al., 1998) have demonstrated differences in the microbial diversity of natural and perturbed environments. These investigators demonstrated that agricultural management, fish farming and pollution lead to profound changes in the soil microbiology, resulting in a reduction in the bacterial diversity. Thus the bacterial fingerprint community of an environment may be disturbed as a result of physico-chemical perturbations. This study presents a fingerprint analysis of dental plaque sampled from the gingival crevice of pre-pubertal children with and without gingivitis. Any marked differences observed in the microbial composition of plaque associated with healthy sites and gingivitis will further advance our understanding of how specific bacteria are associated with, and respond to inflammation and indeed the progression to, periodontal disease.

Analysis of DGGE profiles often involves the use of principle components (Ogino et al., 2001; Widmer et al., 2001) and multidimensional scaling (Iwamoto et al., 2000; Boon et al., 2002), but generally utilises hierarchical cluster analysis to demonstrate similarities in the data and the results are invariably presented in the form of dendrograms (Eichner et al., 1999; Boon et al., 2000, 2002; Van Der et al., 2001). This study analyses the DGGE profiles of the dental plaque sampled from both cohorts by using hierarchical cluster analyses as well as measuring community diversity by means of the Shannon-Wiener index (Shannon and Weaver, 1971). Furthermore, a statistical test for the analysis of the electrophoretic fingerprints has

been applied. This involves a logistic regression analysis that looks at single bands and computes a regression relationship between independent or explanatory variables. This type of analysis can pinpoint individual bands that when present/absent will affect the outcome of interest. In this study the outcomes were (i) whether the subject had gingivitis or not and (ii) the prevalence of the three periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*.

5.2 Materials and methods

5.2.1 Perpendicular DGGE

The DNA extracted from plaque from 20 individuals was pooled and the 16S rRNA gene was partially amplified by touchdown PCR. Equal volumes of cleaned PCR product and loading buffer (final volume of 800 µl) was added to a perpendicular DGGE gel (figure 5.1). This gel had 0-100% denaturant gradient and was electrophoresed for 735 V hrs at 60°C (chapter 2.9). The gels were then stained for an hour and a gel image was then photographed.

5.2.2 Preliminary tests

The oral bacteria *T. forsythensis*, *E. corrodens*, *S. sanguinis* and *A. actinomycetemcomitans* were grown and a DNA extraction was performed. These acted as templates for a touchdown PCR (detailed in section 2.8), the products of which were sequenced. The sequences were aligned using ClustalX (Thompson et al., 1997). The sequences were exported into the WinMelt software (BioRad) to find out the different melting domains of all four sequences and therefore how they would separate on a DGGE gel. This was verified by running the PCR products of all four species on a parallel DGGE gel (detailed in section 2.9).

5.2.3 DGGE profiling

Random DNA from dental plaque sampled from children without gingivitis (group 1 plaque, n = 30) and children with gingivitis (group 2 plaque, n = 30) were used as templates in a touchdown PCR (see section 2.8). In addition, two positive controls consisting of genomic DNA extracted from *T. forsythensis* and *P. intermedia* as well as a negative control consisting of sterile deionised water were included. Individual PCR products and controls were loaded onto parallel gels and electrophoresed (see section 2.9).

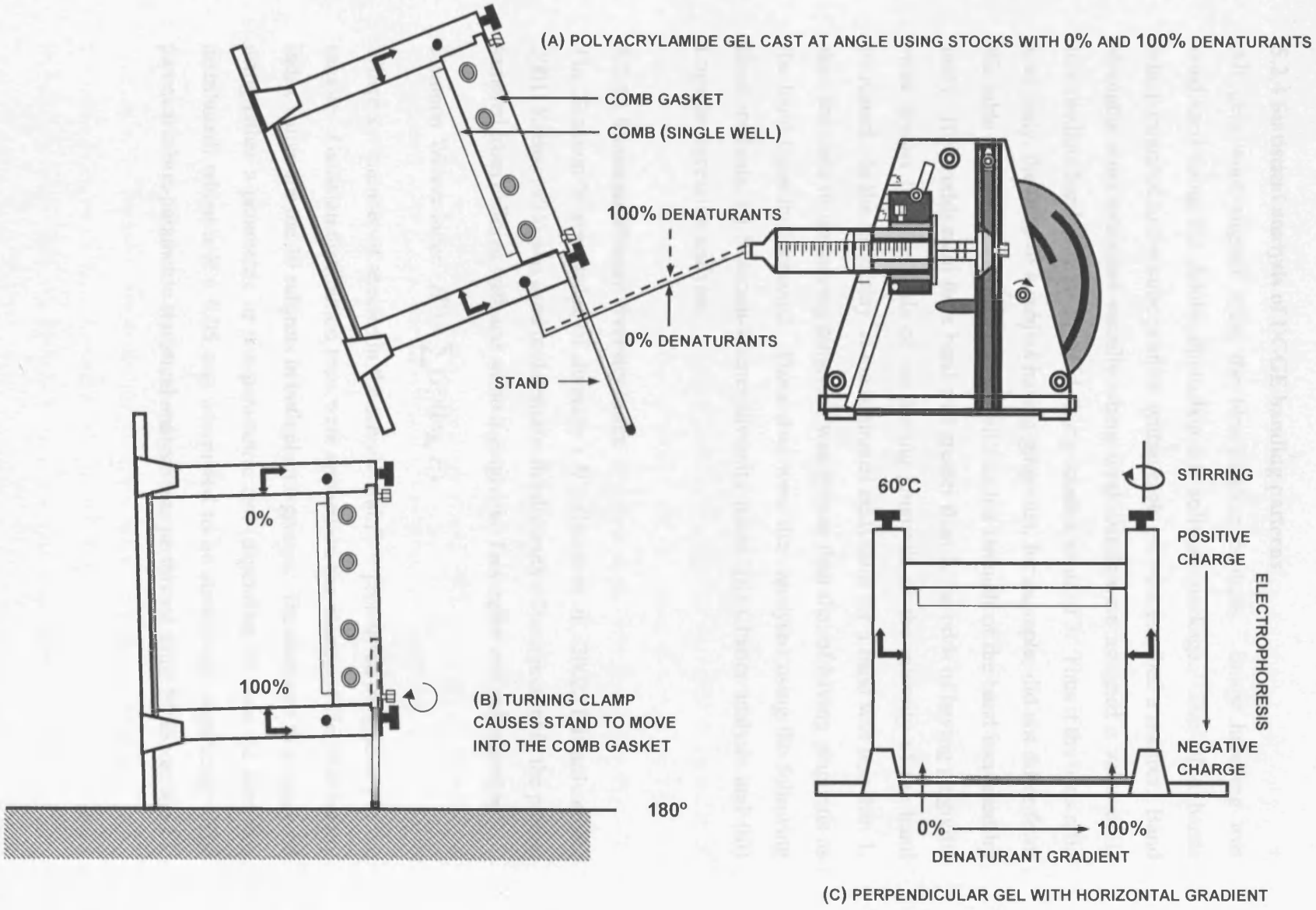


Figure 5.1: System setup for casting perpendicular DGGE gels

5.2.4 Statistical analysis of DGGE banding patterns

All gels were aligned using the two positive controls. Image handling was conducted using the Adobe Photoshop 6.0 software package. The DNA bands which migrated to the same position within each gel were ascribed a number. Band strengths were estimated visually where weak bands were assigned a value of 1, intermediate bands a value of 2 and strong bands a value of 3. Thus if the odds ratio was unity, the odds of a subject having gingivitis, for example, did not differ from the odds of a subject having no gingivitis as the strength of the band increased by unity. If the odds ratio for a band was greater than 1, the odds of having gingivitis were greater than the odds of not having gingivitis as the intensity of the band increased. In the same way, if the estimated odds ratio for a band was less than 1, then the odds of not having gingivitis was greater than that of having gingivitis as the band intensity increased. These data were then analysed using the following three methods; (i) Shannon-Wiener diversity index, (ii) Cluster analysis and (iii) Logistic regression analysis.

5.2.4.1 Shannon-Wiener diversity index

The Shannon-Wiener index of diversity (H') (Boon et al., 2002; Edwards et al., 2001; Krebs, 1985) was used to determine the diversity of taxa present in the plaque sampled from children with and without gingivitis. This index was calculated by:

$$\text{Shannon-Wiener index } (H') = \sum_{i=1}^{s} (P_i)(\log_e P_i)$$

Where s = number of species in the sample and P_i = proportion of species $_i$ in the sample. Gaussian distribution tests were applied on the Shannon-Wiener diversity index values for the 30 subjects in both plaque groups. The data was then analysed using either a parametric or non-parametric test (depending on how the data was distributed) where a $P < 0.05$ was interpreted to be statistically significant. The parametric/non-parametric statistical analysis was performed using SPSS version 12.

5.2.4.2 Cluster analysis

Similarities between the banding patterns generated by PCR-DGGE of the plaque samples were analysed using the Pearson correlation coefficient (Smalla et al., 2001). Similarities were displayed graphically as a dendrogram. The clustering algorithms used to calculate the dendrograms was an unweighted pair group method with arithmetic averages (UPGMA) (Silva and Russo, 2000; Fromin et al., 2002). The cluster analysis and dendrogram generation were carried out by using the Phoretix 1D Advanced and Phoretix 1D Database software (Phoretix International, Newcastle upon Tyne, UK).

5.2.4.3 Logistic regression

(I) For community analysis of subjects with and without gingivitis

The data was first analysed using univariable Chi square tests with a cut-off for significance of 5% to reduce the number of bands to include in the multivariate analysis. The bands demonstrating significance were then included as explanatory variables (each band was recorded as absent = 0 or present = 1) in a multivariate logistic regression analysis, for which the codes, no gingivitis = 0 and gingivitis = 1, were used for the dependent variable. The estimated odds ratio with a 95% confidence interval was calculated for each band in the logistic regression model. The predicted probabilities for a subject belonging to a particular group (in this case gingivitis and no gingivitis) were also calculated. Furthermore, a Hosmer and Lemeshow test for goodness-of-fit of the logistic model was performed. The logistic regression analysis was conducted using SPSS version 12.

(II) For community analysis of subjects harbouring the three periodontal pathogens of interest

The data was first analysed using univariable Chi square tests with a cut-off for significance of 5% to reduce the number of bands to include in the multivariate analysis. The bands demonstrating significance were then included as explanatory variables (each band was recorded as absent = 0 or present = 1) in a multivariate logistic regression analysis, for which the codes, pathogen absent = 0 and pathogen

present = 1, were used for the dependent variable. The estimated odds ratio with a 95% confidence interval was calculated for each band in the logistic regression model. The predicted probabilities for a subject harbouring one of the three pathogens (*P. gingivalis*, *A. actinomycetemcomitans* or *T. forsythensis*) were also calculated. Furthermore, a Hosmer and Lemeshow test for goodness-of-fit of the logistic model was performed.

5.3 Results

5.3.1 Perpendicular DGGE

The perpendicular DGGE profile for pooled plaque shown in figure 5.2 demonstrates the separation and resolution of the different operational taxonomic units (OTUs) from the plaque samples. The greatest vertical separation of the products is between the concentrations of 40-80% of the gradient.

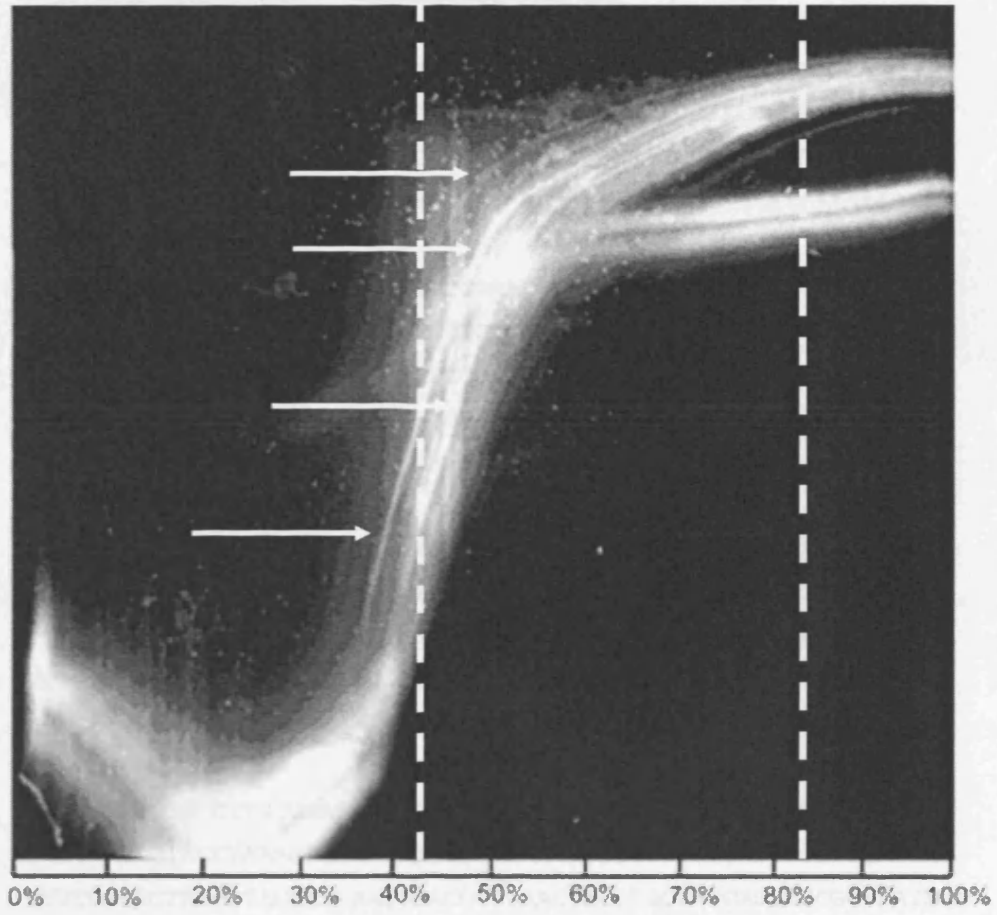


Figure 5.2: Perpendicular DGGE profile from mixed dental plaque microbial communities. A perpendicular DGGE gel in which the denaturing gradient is perpendicular to the direction of electrophoresis. DNA consists of PCR amplified from the 16S rRNA genes of the total bacteria present in the pooled plaque from 20 different children. Dashed lines delineate the 40%-80% boundaries. Arrows demonstrate single bands resolving

5.3.2 Preliminary tests

Multiple alignments of DNA sequences amplified from the same gene from diverse taxa can identify regions of both similarity and variation within the aligned sequences. The multiple alignment with ClustalX demonstrated that there were regions of sequence variations for the four oral bacteria *T. forsythensis*, *E. corrodens*, *S. sanguinis* and *A. actinomycetemcomitans*. This demonstrated that the primers 357FGC and 518R were likely to be suitable for DGGE.

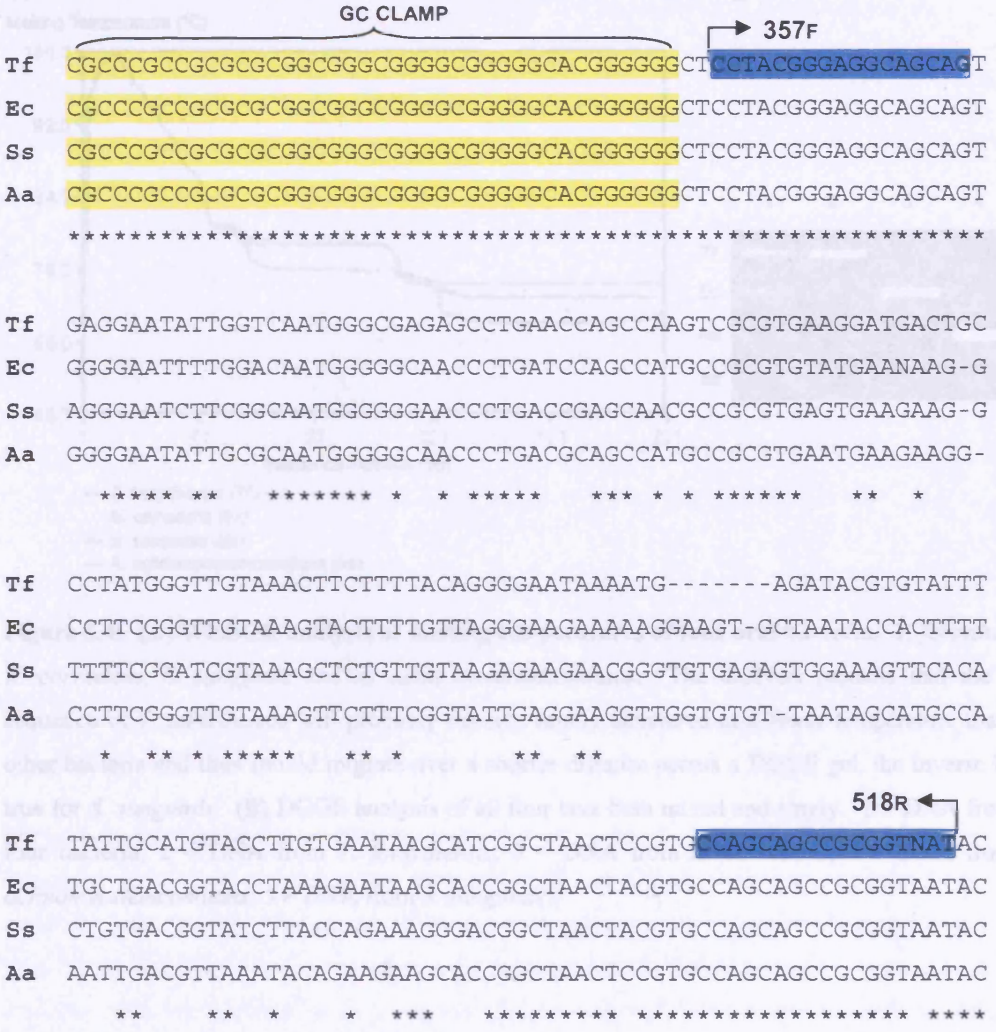


Figure 5.3: Multiple alignments of PCR products amplified from the 16S rRNA genes of four oral bacteria. *T. forsythensis* (**Tf**), *E. corrodens* (**Ec**), *S.s sanguinis* (**Ss**) and *A. actinomycetemcomitans* (**Aa**). The region highlighted in yellow denotes a 40 bp GC clamp. Sequences of homology, at each base position, among all four bacteria are demonstrated with an asterisk (*)

The ability to separate out mixed PCR products amplified from the 16S rRNA genes of these four oral bacteria by DGGE was verified *in silico* by using the WinMelt software package (BioRad). WinMelt results demonstrated that touchdown PCR products for all four bacteria should migrate to different positions in a DGGE gel (figure 5.4). This was then finally confirmed by DGGE (figure 5.4).

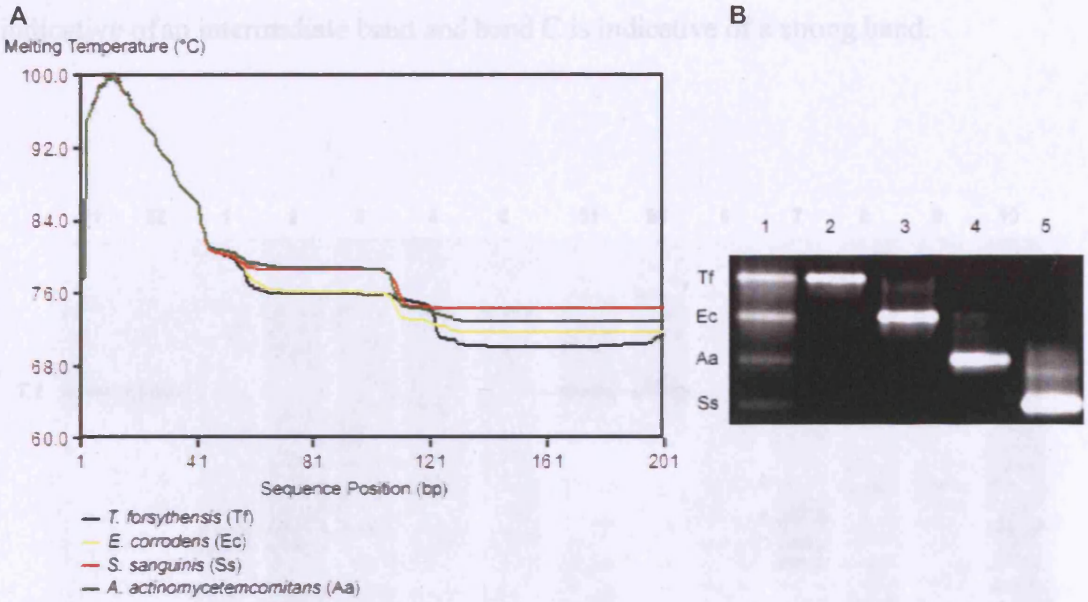


Figure 5.4: (A) WinMelt analysis of melting temperatures of four oral bacteria. *T. forsythensis*, *E. corrodens*, *S. sanguinis* and *A. actinomycetemcomitans*. The analysis predicts that the base sequence of *T. forsythensis* will probably become totally denatured at a lower temperature than the other bacteria and thus should migrate over a shorter distance across a DGGE gel, the inverse holds true for *S. sanguinis*. **(B)** DGGE analysis of all four taxa both mixed and singly. 1 = DNA from all four bacteria; 2 = DNA from *T. forsythensis*; 3 = DNA from *E. corrodens*; 4 = DNA from *A. actinomycetemcomitans*; 5 = DNA from *S. sanguinis*

5.3.3 DGGE profiling

Dental plaque from children with and without gingivitis was subjected to DGGE analysis and provided a complex fingerprint of bands of varying intensities and with different migration distances (figure 5.5). The figure shows four controls (S1 and S2) and 10 plaque sample profiles. Samples 1 and 9 show a relatively simple (but different) profile, while bands 2 and 10 show more complex (and differing) profiles. Band intensities are also illustrated. Band A is indicative of a weak band, band B is indicative of an intermediate band and band C is indicative of a strong band.

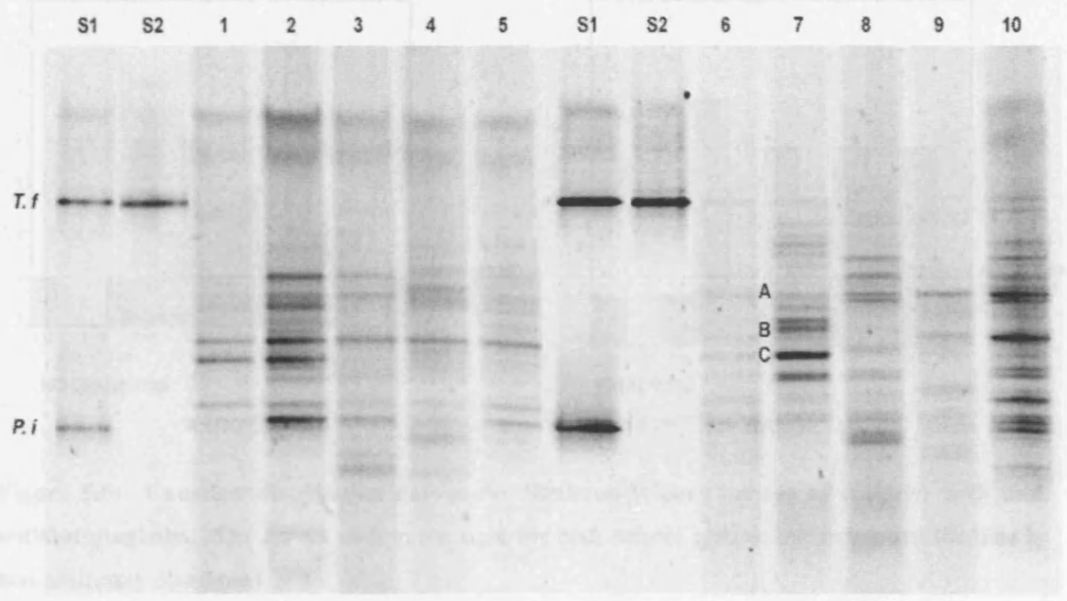


Figure 5.5: Parallel DGGE profiles from 10 different subjects. A parallel DGGE gel in which the denaturing gradient is parallel to the electrophoresis. DGGE profiles were obtained from DNA amplified from the 16S rRNA genes of 10 plaque samples and two controls. Two gel images have been joined together using the control lane S1 with the first gel finishing at lane 5. Lane S1 shows the DNA migratory position for the 2 controls *T. forsythensis* (*T.f*) and *P. intermedia* (*P.i*). Lane S2 demonstrates that DNA from one species migrates to the same position whether it is present by itself or mixed with DNA from another species. Band A is indicative of a weak band, band B is indicative of an intermediate band and band C is indicative of a strong band. Lanes 1-3 and 6-7 demonstrate the banding patterns of plaque from individuals without gingivitis. Lanes 4-5 and 8-10 demonstrate the banding pattern of plaque from individuals with gingivitis

5.3.4 Shannon-Wiener diversity index

The Shannon-Wiener indices for the subjects with and without gingivitis were shown to be non-uniformly distributed by Gaussian distribution curves (figure 5.6). This was evidenced by the curves shifting to the right for both subject groups. These data were therefore compared using the non-parametric Mann-Whitney U Test. The results revealed that there was a significantly greater biological diversity in the sample group with no gingivitis (no-gingivitis group: median = 3.4, range = 2.5 to 4.3; gingivitis group: median = 3.2, range = 1.6 to 4.1; P = 0.009).

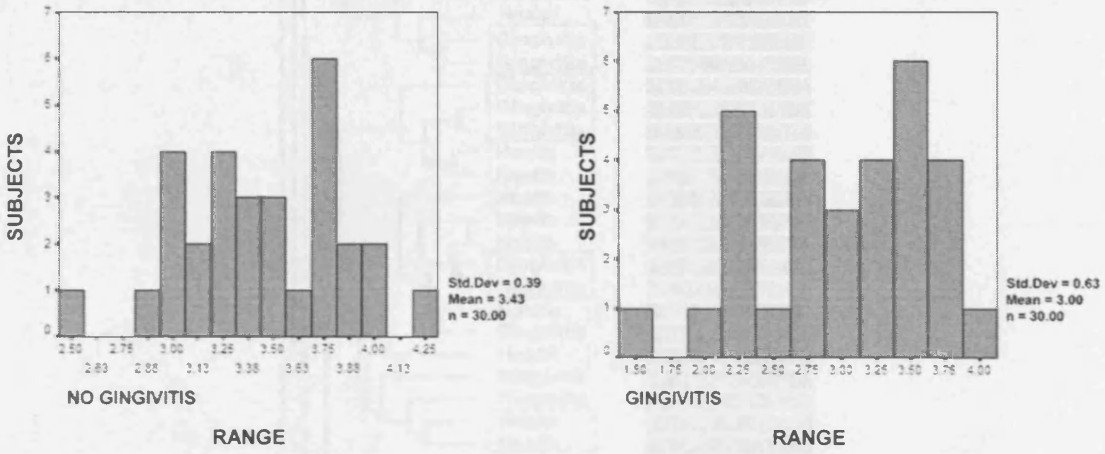


Figure 5.6: Gaussian distribution curves for Shannon-Wiener indices of children with and without gingivitis. The curves shift to the right for both subject groups, indicating that the data is non-uniformly distributed

5.3.5 Cluster analysis

The banding patterns generated were analysed using the Pearson correlation coefficient and displayed graphically as an UPGMA dendrogram generated using the Phoretix software and is shown in figure 5.7. This demonstrates that of the multiple clades resolved 7 were associated with the gingivitis group and 5 associated with the no-gingivitis group.

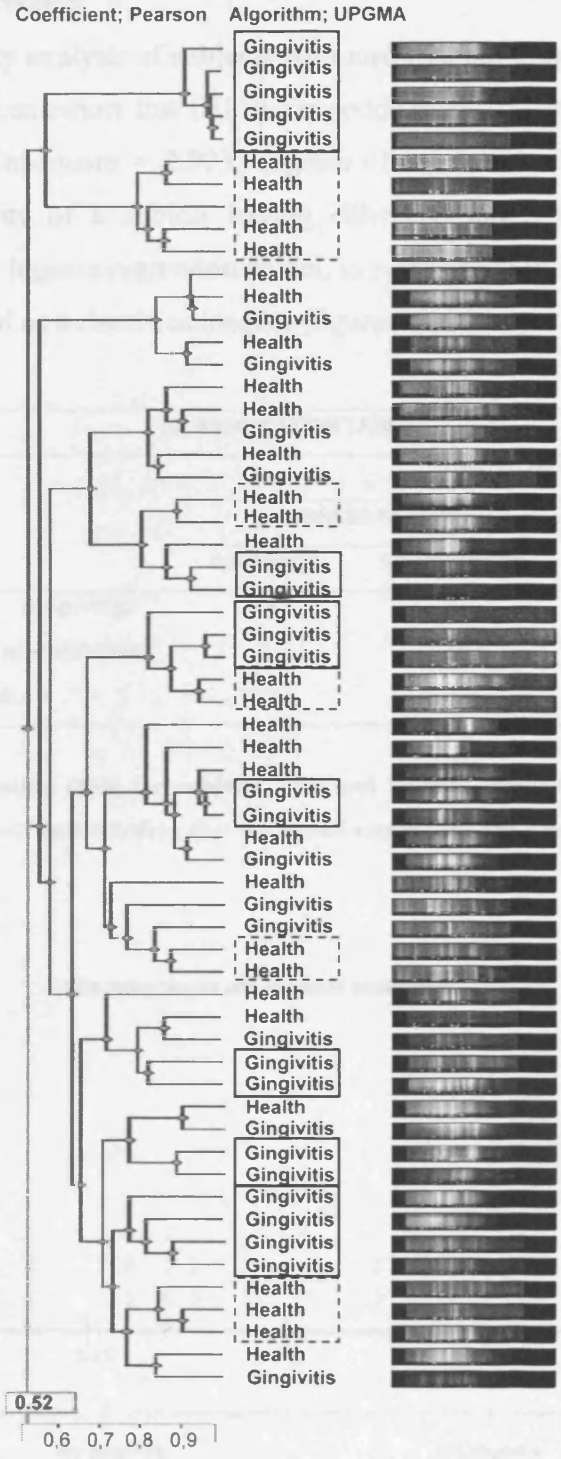


Figure 5.7: Hierarchical cluster analysis of 60 DGGE profiles. Hierarchical cluster analysis results of all the DGGE profiles demonstrated graphically as an UPGMA dendrogram. The diseases status (gingival health [no gingivitis], or gingivitis) and a DGGE fingerprint for all 60 subjects are shown. Solid boxes surround "gingivitis" clades and broken boxes surround "healthy" clades.

5.3.6 Logistic regression

5.3.6.1 Community analysis of subjects with and without gingivitis

The Hosmer and Lemeshow test (HLT) for goodness-of-fit showed that the model was a good fit (Chi-square = 2.903, degrees of freedom = 8, P = 0.940). The expected probability of a subject having either no gingivitis or gingivitis, as determined by the logistic regression model, is represented in both a classification table (table 5.1) and as a classification plot (figure 5.8).

CLASSIFICATION TABLE

OBSERVED		PREDICTED		
		DISEASE STATUS		PERCENTAGE
		GINGIVITIS	NO GINGIVITIS	CORRECT
DISEASE STATUS	GINGIVITIS	24	4	85.7
	NO-GINGIVITIS	6	26	81.3
OVERALL PERCENTAGE				83.5

Table 5.1: Classification table for subjects with and without gingivitis. Classification table (entries are frequencies) demonstrating that the model can predict the correct disease status in an individual

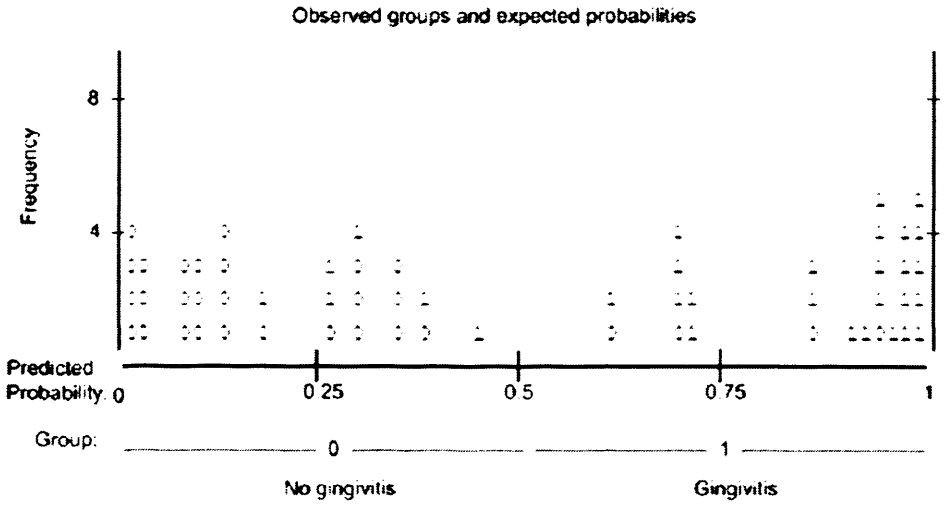


Figure 5.8: Classification plot for subjects with and without gingivitis. Classification plot from the logistic regression analysis. Code 0 = patient with no gingivitis and Code 1 = patient with gingivitis

The classification table (table 5.1) shows that the model works well and approximately 84% of the individuals were correctly classified as having the appropriate inflammatory status. The classification plot (figure 5.8) further demonstrates that the model works well in that most subjects with no gingivitis (code = 0) are predicted as having no gingivitis (i.e. on the left hand side of the horizontal axis). Likewise, most of the subjects with gingivitis (code = 1) are predicted as having gingivitis (i.e. on the right hand side of the horizontal axis).

Logistic regression analysis also determined that three bands were significantly associated with the presence or absence of gingivitis in these subjects. From this regression analysis, the estimated odds ratio of any band could be interpreted as the ratio of the odds of having gingivitis in those subjects with the band to the odds of having gingivitis in those subjects without the band. Table 5.2 presents the results of the analysis and demonstrated that band 2 was significantly associated with no gingivitis (P = 0.001), whilst bands 1 and 3 were significantly associated with gingivitis (P = 0.042, P = 0.005). The positions of these three bands on a DGGE gel are shown in figure 5.9).

BAND	CLUSTER ANALYSIS		WALD TEST STATISTIC	DF	P VALUE	ODDS RATIO	95% CI	
	MODEL COEFFICIENT	SE					LOWER	UPPER
1	2.272	1.117	4.135	1	0.042	9.699	1.085	86.656
2	-3.274	1.026	10.192	1	0.001	0.038	0.005	0.282
3	2.904	1.035	7.865	1	0.005	18.247	2.398	138.868
CONSTANT	-1.976	0.964	4.205	1	0.040	0.139		

Table 5.2: Logistic regression analysis results for subjets with and without gingivitis. Logistic regression analysis results indicating which bands were significantly associated with gingivitis ("gingivitis" and "no gingivitis" were coded as 0 and 1 respectively; DF = degrees of freedom; 95% CI = 95% confidence interval)

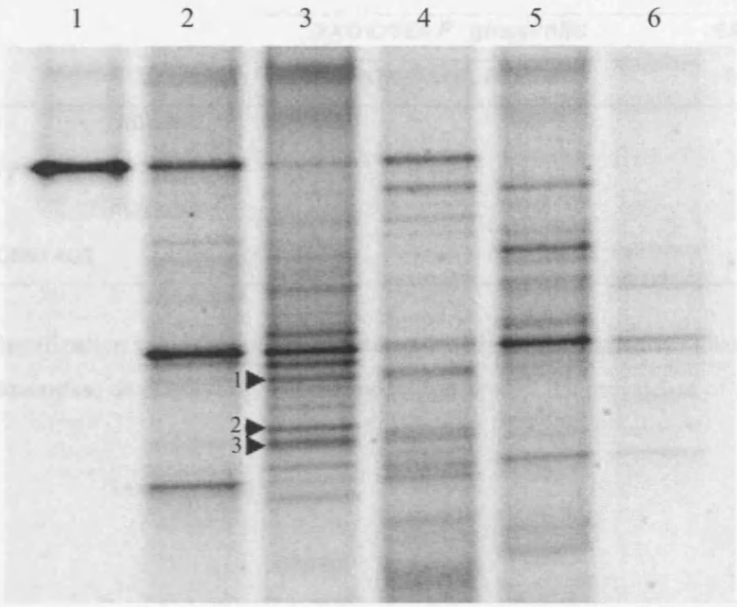


Figure 5.9: DGGE bands of interest for sequencing. DGGE profiles of plaque from five children demonstrating bands that were significantly associated with health or gingivitis. Band 2 is associated with no gingivitis and bands 1 and 3 are associated with gingivitis.

5.3.6.2 Community analysis of subjects harbouring the three periodontal pathogens of interest

(I) *P. gingivalis*: The HLT for goodness-of-fit showed that the model was a good fit for *P. gingivalis* (Chi-square = 6.054, degrees of freedom = 8, P = 0.641). The expected probability of a subject having *P. gingivalis*, as determined by the logistic regression model, is represented in both a classification table (table 5.3) and as a classification plot (figure 5.10). The classification table (table 5.3) shows that the model works well and 86.7% of the individuals were correctly identified as harbouring *P. gingivalis*. This can be observed from the classification plot (figure 5.10), were subjects with no *P. gingivalis* (code = 0) are predicted as having no *P. gingivalis* (i.e. on the left hand side of the horizontal axis). Likewise, most of the subjects with *P. gingivalis* (code = 1) are predicted as having *P. gingivalis* (i.e. on the right hand side of the horizontal axis).

CLASSIFICATION TABLE

OBSERVED		PREDICTED		
		PATHOGEN *P. gingivalis*		PERCENTAGE
		ABSENT	PRESENT	CORRECT
PATHOGEN *P. gingivalis*	ABSENT	30	4	88.2
	PRESENT	4	22	84.6
OVERALL PERCENTAGE				86.7

Table 5.3: Classification table for subjects with and without *P. gingivalis*. Classification table (entries are frequencies) demonstrating that the model can predict the prevalence of *P. gingivalis* in an individual

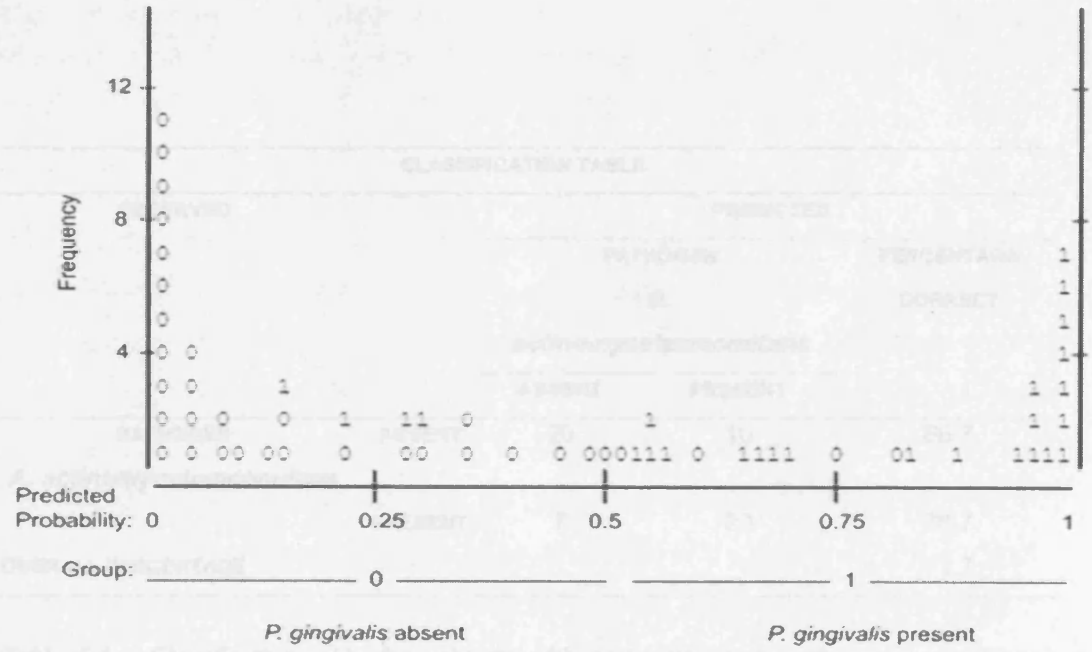


Figure 5.10: Classification plot for subjects with and without *P. gingivalis*. Classification plot from the logistic regression analysis. Code 0 = patient with no *P. gingivalis* and Code 1 = patient with *P. gingivalis*

(II) ***A. actinomycetemcomitans*:** The HLT for goodness-of-fit showed that the model was a reasonable fit for *A. actinomycetemcomitans* (Chi-square = 11.359, degrees of freedom = 8, P = 0.182). The expected probability of a subject having *A. actinomycetemcomitans*, as determined by the logistic regression model, is represented in both a classification table (table 5.4) and as a classification plot (figure 5.11). The classification table (table 5.4) shows that the model works well and 71.7% of the individuals were correctly identified as harbouring *A. actinomycetemcomitans*. This can be observed from the classification plot (Figure 5.11), where subjects with no *A. actinomycetemcomitans* (code = 0) are predicted as having no *A. actinomycetemcomitans* (i.e. on the left hand side of the horizontal axis). Likewise, most of the subjects with *A. actinomycetemcomitans* (code = 1) are predicted as having *A. actinomycetemcomitans* (i.e. on the right hand side of the horizontal axis).

CLASSIFICATION TABLE				
OBSERVED		PREDICTED		
		PATHOGEN *A. actinomycetemcomitans*		PERCENTAGE CORRECT
		ABSENT	PRESENT	
PATHOGEN *A. actinomycetemcomitans*	ABSENT	20	10	66.7
	PRESENT	7	23	76.7
OVERALL PERCENTAGE				71.7

Table 5.4: Classification table for subjects with and without *A. actinomycetemcomitans*. Classification table (entries are frequencies) demonstrating that the model can predict the prevalence of *A. actinomycetemcomitans* in an individual

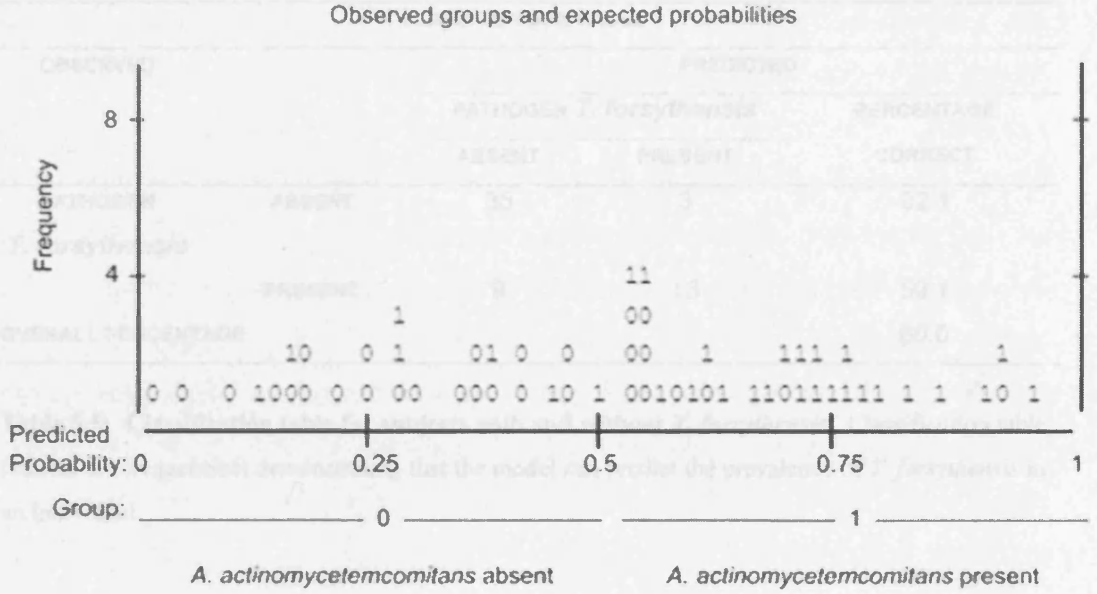


Figure 5.11: Classification plot for subjects with and without *A. actinomycetemcomitans*. Classification plot from the logistic regression analysis. Code 0 = patient with no *A. actinomycetemcomitans* and Code 1 = patient with *A. actinomycetemcomitans*

(II) *T. forsythensis*: The HLT for goodness-of-fit showed that the model was a good fit for *T. forsythensis* (Chi-square = 5.389, degrees of freedom = 8, P = 0.715). The expected probability of a subject having *T. forsythensis*, as determined by the logistic regression model, is represented in both a classification table (table 5.5) and as a classification plot (figure 5.12). The classification table (table 5.5) shows that the model works well and 80% of the individuals were correctly identified as harbouring *T. forsythensis*. This can be observed from the classification plot (figure 5.12), were subjects with no *T. forsythensis* (code = 0) are predicted as having no *T. forsythensis* (i.e. on the left hand side of the horizontal axis). Likewise, most of the subjects with *T. forsythensis* (code = 1) are predicted as having *T. forsythensis* (i.e. on the right hand side of the horizontal axis).

CLASSIFICATION TABLE				
OBSERVED		PREDICTED		
		PATHOGEN *T. forsythensis*		PERCENTAGE
		ABSENT	PRESENT	CORRECT
PATHOGEN *T. forsythensis*	ABSENT	35	3	92.1
	PRESENT	9	13	59.1
OVERALL PERCENTAGE				80.0

Table 5.5: Classification table for subjects with and without *T. forsythensis*. Classification table (entries are frequencies) demonstrating that the model can predict the prevalence of *T. forsythensis* in an individual

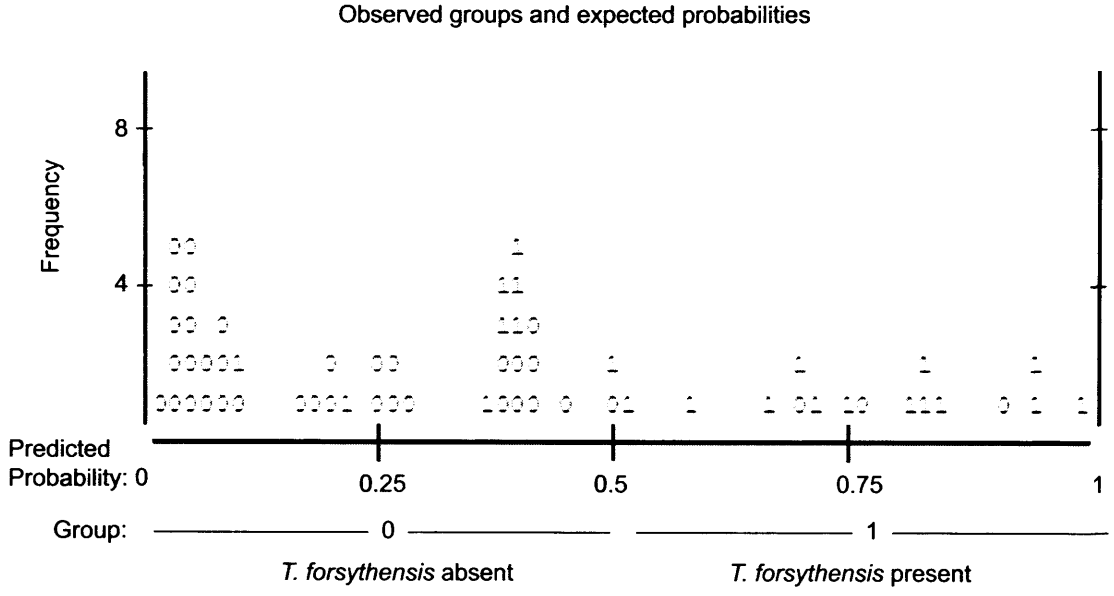


Figure 5.12: Classification plot for subjects with and without *T. forsythensis*. Classification plot from the logistic regression analysis. Code 0 = patient with no *T. forsythensis* and Code 1 = patient with *T. forsythensis*

Logistic regression analysis also determined that two bands were significantly associated with the presence of *P. gingivalis* in these subjects, whilst one band was significantly associated with the presence of *A. actinomycetemcomitans*. No DGGE bands were observed that influenced the prevalence of *T. forsythensis*. From this regression analysis, the estimated odds ratio of any band could be interpreted as the ratio of the odds of harbouring these pathogens in those subjects with the band to the odds of harbouring these pathogens in those subjects without the band. Tables 5.6 and 5.7 present the results of the analysis and demonstrated that bands 4 and 6 were significantly associated with the presence of *P. gingivalis* (P = 0.027, P = 0.038) whilst band 5 (P = 0.035) was significantly associated with the presence of *A. actinomycetemcomitans*. The position of these three bands on a DGGE gel is shown on figure 5.13.

BAND	CLUSTER ANALYSIS		WALD TEST STATISTIC	DF	P VALUE	ODDS RATIO	95% CI	
	MODEL COEFFICIENT	SE					LOWER	UPPER
4	4.102	1.849	4.922	1	0.027	60.489	1.613	2268.198
6	5.436	2.626	4.287	1	0.038	229.603	1.336	39448.163
CONSTANT	10.349	60.535	0.029	1	0.864	31234.9		

Table 5.6: Logistic regression analysis for subjects with and without *P. gingivalis*. Logistic regression analysis results indicating which bands were significantly associated with the prevalence of *P. gingivalis* ("absence" and "presence" were coded as 0 and 1 respectively; DF = degrees of freedom; 95% CI = 95% confidence interval)

BAND	CLUSTER ANALYSIS		WALD TEST STATISTIC	DF	P VALUE	ODDS RATIO	95% CI	
	MODEL COEFFICIENT	SE					LOWER	UPPER
5	2.200	1.046	4.421	1	0.035	9.02	1.161	70.186
CONSTANT	-8.717	36.746	0.056	1	0.812	0		

Table 5.7: Logistic regression analysis for subjects with and without *A. actinomycetemcomitans*. Logistic regression analysis results indicating which bands were significantly associated with the prevalence of *A. actinomycetemcomitans* ("absence" and "presence" were coded as 0 and 1 respectively; DF = degrees of freedom; 95% CI = 95% confidence interval)

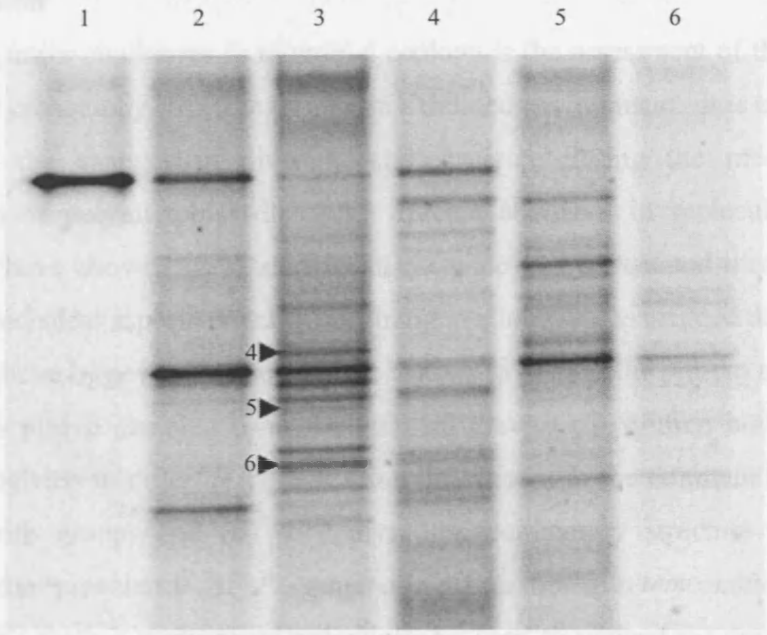


Figure 5.13: DGGE bands associated with the prevalence of *P. gingivalis* and *A. actinomycetemcomitans*. DGGE profiles of plaque from 5 children demonstrating bands that were significantly associated with the prevalence of *P. gingivalis* and *A. actinomycetemcomitans*. Bands 4 and 6 are associated with the presence of *P. gingivalis*, whilst band 5 is associated with the presence of *A. actinomycetemcomitans*. No bands were associated with the presence of *T. forsythensis*

5.4 Discussion

One of the major challenges in microbial ecology is the assessment of the bacterial diversity or community structure present in a defined environment. It is important to understand the changes in diversity and structure during the inception and progression of polymicrobial diseases. Recent advances in molecular biology techniques have allowed us to start to characterise and understand these changes. While the technical aspects are now becoming available the associated data analysis techniques have lagged behind somewhat. A case in point is the present study which investigates plaque sampled from the gingival crevice of children both with and without gingivitis in order to (i) detect any differences in the community structure between both groups and (ii) to analyse the community structure that might influence the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Such a study could potentially be used to understand the differences between a healthy dental microbiota and its progression to periodontal disease. A culture independent technique (DGGE) was used to characterise the population differences between the two cohorts in an effort to include the uncultivable portion of the oral microbiota in the analysis. The advantages of using DGGE as a means of studying microbial ecology are well established in that it provides a more accurate means of visualising whole microbial communities (since there should be a reduced bias in the detection of unculturable species) and it is less labour intensive than the more conventional technique of PCR-cloning and sequencing. The results obtained from the perpendicular DGGE revealed that the best concentration gradient of denaturants to work with, when analysing dental plaque communities, was 40-80%. All DGGE polyacrylamide gels thus had a gradient running from 40% to 80% in strength.

Once the data were generated for all the subjects in both cohorts a number of different analysis techniques were used to interpret these data. This included a "by eye" analysis as reported by other workers (Tannock, 2002; McBain et al., 2003). It is relatively easy to comment on three or four patterns and select bands of interest based on visual analysis. The banding patterns generated were quite complex and

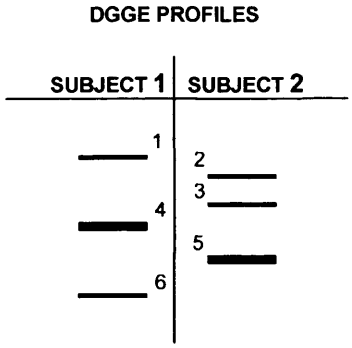
comparing 60 patterns with each other proved difficult. Initially the data were analysed using a similar approach commonly used by other workers e.g. diversity indices and cluster analysis.

There are various ecological diversity measures but their suitability for use with highly varied microbial communities is unclear (Hill et al., 2003). The Shannon-Wiener index of diversity (Shannon and Weaver, 1971) has been previously used in microbial ecology (Edwards et al., 2001; Ogino et al., 2001; Hill et al., 2003; Muller et al., 2003). There is some confusion surrounding this index, as exemplified by it being referred to by different names. The full title of this index is the Shannon-Wiener function, after Claude Shannon and Norbert Wiener (Shannon and Weaver, 1971). It is more commonly known as the Shannon-Weaver index after Shannon's co-author, Wallace Weaver. The Shannon-Wiener index of diversity was applied to the fingerprint patterns generated by DGGE. By using both the total number of DGGE bands and their relative intensities, it was possible to calculate the bacterial diversity index (H'). This should provide a measure of sample diversity without the need for cultivation. It was assumed that not all the DNA within the sample would be amplified sufficiently to be visualised as bands. Thus DGGE fingerprints would reflect the most abundant rDNA types from dental plaque or perhaps those strains most applicable to amplification (Polz and Cavanaugh, 1998). Thus the diversity indices calculated from the DGGE pattern were regarded as relative. Earlier workers (Eichner et al., 1999) have demonstrated that the diversity index H' can be applied to complex microbial communities and is well suited for comparing large sets of samples.

The results demonstrated that there was a greater biological diversity in the sample group with no gingivitis than the gingivitis group ($P = 0.009$). This might indicate that a decrease in bacterial diversity may be associated with the shift from health to gingivitis. According to the 'ecological plaque hypothesis' (Marsh and Martin, 1999) a change in the environment leads to a shift on the community structure. This shift is only a change in proportions of certain taxa within the community. Perhaps

the perceived decrease in diversity is as a result of certain taxa proliferating as a result of inflammation and as a consequence "masking" the presence of bacteria that do not necessarily increase in number during the inflammation. It can be postulated that the observed reduction in bacterial diversity in gingivitis may arise as a decreased ability to detect other bacterial taxa that are commonly present in health. These taxa will be present in lower numbers compared to those that have proliferated during the inflammatory process. Nevertheless, when considering ecological diversity and community structure it is believed that species diversity is an important feature in maintaining a degree of stability within that community. Thus it is expected that stable and resilient microbial communities must contain a certain level of diversity (Atlas, 1994). As has been shown the present results demonstrate a greater diversity in the DGGE profiles of dental plaque associated with no gingivitis. This may indicate that the dental plaque community associated with no gingivitis is probably more stable than the dental plaque community associated with gingivitis. While the overall precepts of ecology support this (Atlas, 1994), workers using culture-dependent techniques to study experimental gingivitis have come to the opposite conclusion (Moore et al., 1982). There are no studies which are experimentally comparable to this present study and thus detailed comparisons are precluded. A factor which may go some way to reconciling these apparently conflicting results is that the DGGE technique takes into account the uncultivable microbiota present in the sample (in this case the oral cavity where it is thought that around 50% of the species are uncultivable) (Paster et al., 2001).

The use of diversity indices for DGGE analysis in this case uses very little of the data present and as stated only provides a measure of diversity. It is entirely possible for two communities to be equally diverse (i.e. equal H') but have, in the most extreme case, a completely different species structure (Atlas, 1994) (figure 5.14). It is likely that the DGGE data contains more information than simply this.



DGGE BANDS	BANDS PRESENT IN SUBJECT 1	BANDS PRESENT IN SUBJECT 2
1	1	0
2	0	1
3	0	1
4	2	0
5	0	2
6	1	0

H' = 1.5 FOR BOTH SUBJECTS

Figure 5.14: Schematic representation of different DGGE profiles with the same richness and diversity. Diagram shows a representation of two different DGGE profiles with respect to migration distance and relative intensity. The patterns are clearly different yet the richness (n) and diversity (*H'*) are the same for both patterns

Characteristic profiles of microbial communities or DNA fingerprints can be produced by DGGE. These microbial fingerprints can be represented as binary vectors, which have the potential to be of a very high dimension depending on the environment sampled, such as soil (Torsvik et al., 1990) or dental plaque (Paster et al., 2001). Earlier workers have used principle components (Ogino et al., 2001; Widmer et al., 2001) as well as multidimensional scaling (Iwamoto et al., 2000; Boon et al., 2002) to analyse groups of similarities in the data. Artificial neural networks (ANNs) may provide an alternative to logistic regression analysis in the recognition of DGGE patterns. ANNs detect nonlinear relationships, allow the visualisation of complex data and remain robust despite experimental variations (Dollhopf et al., 2001). Kohonen self organizing maps (SOMs) have been previously used in biological research for pattern recognition (Dollhopf et al., 2001). The use of SOMs might be an interesting technique to apply on the DGGE data generated from subjects with and without gingivitis. Nevertheless, logistic regression analysis generates the same type of outcome without the need for complex software. In general most researchers use hierarchical clustering algorithms based on similarity indices for binary vectors and the results are

invariably presented in the form of dendrograms (Eichner et al., 1999; Boon et al., 2000). Cluster analysis, also known as 'classification', has been defined as the search for a natural grouping (Marriot, 1974). Cluster analysis, for example the UPGMA applied in this study, can be used to identify samples that generate similar patterns (Ibekwe et al., 2001; Boon et al., 2002). The UPGMA dendrogram calculated demonstrated similarities in the banding profiles of plaque sampled from subjects both with and without gingivitis. There were 7 clades that clustered similar band profiles from plaque sampled from gingivitis sites. Likewise the cluster analysis demonstrated 5 clades of similar band profiles from plaque sampled from sites without gingivitis. Indeed 14/30 "Health" samples and 20/30 "Gingivitis" samples clustered together using this method. This implies that specific samples, whether derived from health or gingivitis, are related to each other based on the DGGE banding pattern. It also shows that more than one profile is associated with either condition and therefore that perhaps there are a number of specific microbiotas associated with health or gingivitis. Indeed, while all the gingivitis clades are clearly from gingivitis samples perhaps only one specific clade (with a defined and characteristic microbiota) will eventually lead to periodontitis. Earlier workers have postulated the association of certain taxa with the onset and progression of periodontal disease, such as the red complex (*P. gingivalis, T. forsythensis* and *T. denticola*) (Ximenez-Fyvie et al., 2000a). It was conceivable that perhaps these three key taxa were only represented in one of the gingivitis clades and thus only one type of gingivitis (one type of bacterial community) might actually lead to periodontitis. When further analysed, there appeared to be no correlation between the presence of *P. gingivalis* and *A. actinomycetemcomitans* and the presence/absence of gingivitis in any singular clade. It would be interesting to follow these subjects and monitor their profile and record which clades lead to the inception of periodontal disease. This analysis technique is limited because it does not take into consideration the outcome, either gingivitis or no gingivitis. It aims to look at clusters of bands to predict the outcome but does not utilise the outcome itself when comparing the data for similarities. Earlier workers (Zoetendal et al., 1998; Vanhoutte et al., 2004a) applying similar techniques have demonstrated that

individuals have their own unique faecal microbial community that remains relatively stable over time. This appears to also be the case within the oral cavity (unpublished data). Analysis of the differences in microbial fingerprints for both gingivitis and no gingivitis by cluster analysis may be hampered by the fact that substantial differences may exist within the individual cohorts to start with. The hierarchical cluster analysis is a good technique for longitudinal data but with cross-sectional data there seems to be too much variation within each group.

Both these analyses have yielded valuable information yet further information can be extracted from these DGGE profiles. For example:

(i) Is there a recognisable fingerprint which can be used to differentiate between the gingivitis and no gingivitis group?
(ii) Are there certain bands associated with health or indeed gingivitis?
(iii) Are there certain bands that influence the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*?

A different type of analysis, logistic regression, was used to answer these questions. As opposed to hierarchical cluster analysis, logistic regression analysis takes the outcome into consideration (i.e. gingivitis or no gingivitis as well as the presence or absence of the three pathogens of interest) in addition to differences in band numbers and band migration position. This type of analysis does not account for clusters of multiple bands in an attempt to examine DGGE profiles for similarities. Indeed, it analyses the fingerprint with respect to the presence/absence of individual bands. Analysis of individual bands from the total fingerprint allows for the computation of a regression relationship between a clinical descriptor such as gingival status or the prevalence of a pathogen (outcome variable) and band presence/absence (explanatory variable). The results of the regression analysis have demonstrated that:

(i) There were substantial differences in the DGGE profiles of both cohorts of children. The presence or absence of gingivitis was correctly classified in 83.5% of the subjects. This classification plot (figure 5.8) correctly predicted most subjects with no gingivitis (code = 0) as having no gingivitis and most subjects with gingivitis (code = 1) as having gingivitis based on the presence/absence of bands. Moreover, the results from the regression analysis have further demonstrated that band 2 (figure 5.9) is statistically associated with the absence of gingivitis, whilst bands 1 and 3 were significantly associated with gingivitis.

(ii) The presence of *P. gingivalis, A. actinomycetemcomitans* and *T. forsythensis* were correctly classified respectively in 87.7%, 71.7% and 80% of the subjects. The classification plots (figures 5.10-5.12) correctly predicted most subjects without the pathogen of interest (code = 0) as not harbouring the pathogen. On the other hand, most subjects with the pathogen of interest (code = 1) were correctly predicted to harbour said pathogen based on the presence/absence of bands. Moreover, the results from the regression analysis have also demonstrated that bands 4 and 6 (figure 5.13) were statistically associated with the presence of *P. gingivalis*, whilst band 5 was significantly associated with the presence of *A. actinomycetemcomitans*. No specific bands were significantly associated with the presence/absence of *T. forsythensis*.

DGGE and logistic regression analysis of these 60 subjects have demonstrated that individual operational taxonomic units (OTUs) were significantly associated in influencing the gingival health status of the subjects as well as influencing the prevalence of the three pathogens of interest. The next step involves the identification of these OTU's by DNA sequencing.

5.5 Conclusion

A number of different techniques were used to analyse DGGE banding profiles, each of which answer different and progressively more complex questions. The two analyses used first, the diversity index and the cluster analysis, both provide useful data but do not utilise all available information. The diversity index simply measures a change in diversity but does not specify what changes, or if the community structure is similar. Cluster analysis attempts to differentiate between cohorts that contain large differences within the cohort itself. Furthermore, cluster analysis does not specify which OTUs are important, only that differences exist. Logistic regression not only successfully differentiates between the profiles of both cohorts but can also specify individual OTUs associated with these differences. Logistic regression analysis is the method of choice to analyse and compare DGGE-generated community profiles in cross-sectional studies of complex microbial populations.

Chapter 6:
Sequence analysis of DGGE bands

6.1 Introduction

The DGGE fingerprint analysis of dental plaque samples (chapter 5) demonstrated that five specific OTUs were significantly associated with the presence or absence of gingivitis as well as influencing the prevalence of *P. gingivalis* and *A. actinomycetemcomitans* (bands 3 and 6 are the same band). PCR-sequencing these OTUs should provide the relevant nucleotide sequence information required to obtain an approximate identification for all five bands. Identifying these OTUs might further help in the characterisation of specific taxa that might be implicated in the inception and indeed the progression of gingivitis. Identifying the OTUs that were significantly associated with the presence of *P. gingivalis* and *A. actinomycetemcomitans* could help to further categorise other oral bacteria that might modulate the proliferation and stability of these two putative periodontal pathogens in the oral cavity.

Bands of interest from other work have been previously excised and directly PCR-amplified and sequenced (Ovreas et al., 1997; Ampe et al., 1999; Ercolini et al., 2003) or PCR-cloned and sequenced DGGE bands to successfully identify the taxonomic units of interest (Zwart et al., 1998; Iwamoto et al., 2000). Conversely, recent investigators (Kowalchuk et al., 1997; van Beek and Priest, 2002; Ercolini et al., 2003, 2004) have reported that band excision and sequencing of DGGE bands might not provide unequivocal identifications as a result of the co-migration of DNA fragments from different taxa to the same positions within DGGE gels. Other workers assessing bacterial DNA sequences of excised DGGE bands have also reported that co-migration implies sequence identity (Miambi et al., 2003).

The aim of this analysis was to determine the identity of these five OTUs to further characterise the taxa that might be implicated in gingivitis, as well as observing which taxa are significantly associated with *P. gingivalis and A. actinomycetemcomitans*.

6.2 Materials and methods

6.2.1 DGGE profiling

Dental plaque samples that were known to contain the five OTUs (figure 5.9) were reamplified by touchdown PCR (see section 2.8). Bands of interest from different subjects were confirmed as having migrated to equivalent positions within DGGE gels by using the online tool EquiBands (Huber and Peduzzi, 2004). Pairs of bands migrating to the same position in the DGGE gel were designated as the internal standards. The migration distances for both the internal standards as well as the bands under test were measured (in pixels) using the Adobe Photoshop 6.0 software. These distances were entered into the EquiBands applet (Huber and Peduzzi, 2004). A 'divergence from optimum' value < 3 pixels would demonstrate that the bands being tested were indeed equivalent.

6.2.2 DGGE band excision and PCR-cloning

Three equivalent DGGE bands from different children were excised for the five OTUs. All excised DGGE fragments were electrophoresed through a second, identical DGGE gel, in order to verify whether single DGGE bands had been excised. The excised bands were eluted (see section 2.11) and the DNA was primarily used as template for a direct sequencing reaction (see section 2.12). The excised and eluted DNA was also used in a touchdown PCR using the 357F (no GC clamp) and 518R primers (universal 16S rRNA gene primers). The PCR products were then cloned (see section 2.14) and 20 individual clones for each of the excised DGGE bands were sub-cultured onto LB agar.

6.2.3 PCR-sequencing of clones

A total of five clones, from all three equivalent bands, for each of the five OTUs of interest were PCR-sequenced. Thus a total of 15 clones were sequenced for each OTU. The final reaction volume for each PCR was 50 µl. Master mixtures contained 5 mM 10 x PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM of both primers M13F and M13R and 5U of Taq DNA polymerase. All dNTP's were used at a final concentration of 0.2 mM. Templates consisted of a single colony picked using a

sterile wooden toothpick. The cycling parameters consisted of 30 cycles of: 94°C for 1 min (except 5 min for the first cycle), 54°C for 1 min and 72°C for 1.5 min (except for 5 min for the last cycle). Each PCR was carried out with a negative control consisting of sterile de-ionised water in addition to a positive control consisting of DNA extracted from a pure culture of *Esherichia coli* (10 ng μl^{-1}). The expected product length for this PCR was ca. 370 bp. Smaller fragments were exclusively present in some lanes and indicated a failed ligation reaction and these were not used further. The correctly sized PCR products were cleaned and subjected to a sequencing reaction. The final reaction volume for the reaction was 10 µl. Master mixtures contained 1.5 µM of the T3 primer and 6 µl of Quickstart mix (Beckman Coulter, High Wycombe, UK). The template consisted of the cleaned PCR product at a concentration of 5-500 ng DNA. The cycling parameters carried out on an UNO II thermal cycler (Biometra, Goettingen, Germany) consisted of 30 cycles of: 96°C for 20 s, 50°C for 20 s and 60°C for 4 min. Reactions were carried out on 96 V-bottom thermal cycler-compatible polypropylene plates (Beckman Coulter). The products were then cleaned up according to the manufacturer's instructions (Beckman Coulter) and analysed using a CEQ 8000 DNA analysing system (Beckman Coulter).

6.2.4 Dendrogram generation

Sequenced 16S rRNA gene fragments were compared to other sequences on public databases using basic local alignment search tool (BLAST) (Altschul et al., 1990) to obtain an approximate identification for the originating organism. Sequences were aligned using ClustalX (Thompson et al., 1997), all gaps were removed, and neighbour-joining trees were generated using the PHYLIP software package (Felsenstein, 1993). Dendrograms were used to aid visual interpretation of the phylogenetic relationship between the sequences, and the alignments and distance matrices used to generate the dendrograms were also consulted directly. Each unique sequence was identified based upon the above analyses, and these were used to produce a dendrogram showing the relatedness of taxonomic units detected for each band of interest.

6.3 Results

6.3.1 DGGE profiling

The migration distance of the internal standards (in red) as well as the bands of interest (in black, figure 6.1 A), were entered into the EquiBands applet. The EquiBands applet demonstrated that in all cases the bands of interest gave a divergence from optimum value of less than 3 and thus were equivalent to one another. A typical output of the applet can be observed in figure 6.1 B. This outcome demonstrates a regression plot with the migration distance of the internal standards (in red) and the migration distance of the bands of interest (in black). The migration distance of 955 and 943 pixels, for the bands of interest, are linear with the migration distance of the internal standards. Indeed, the divergence from the linear plot is equivalent to only 1 pixel. Thus the 2 bands tested can be regarded as equivalent bands.

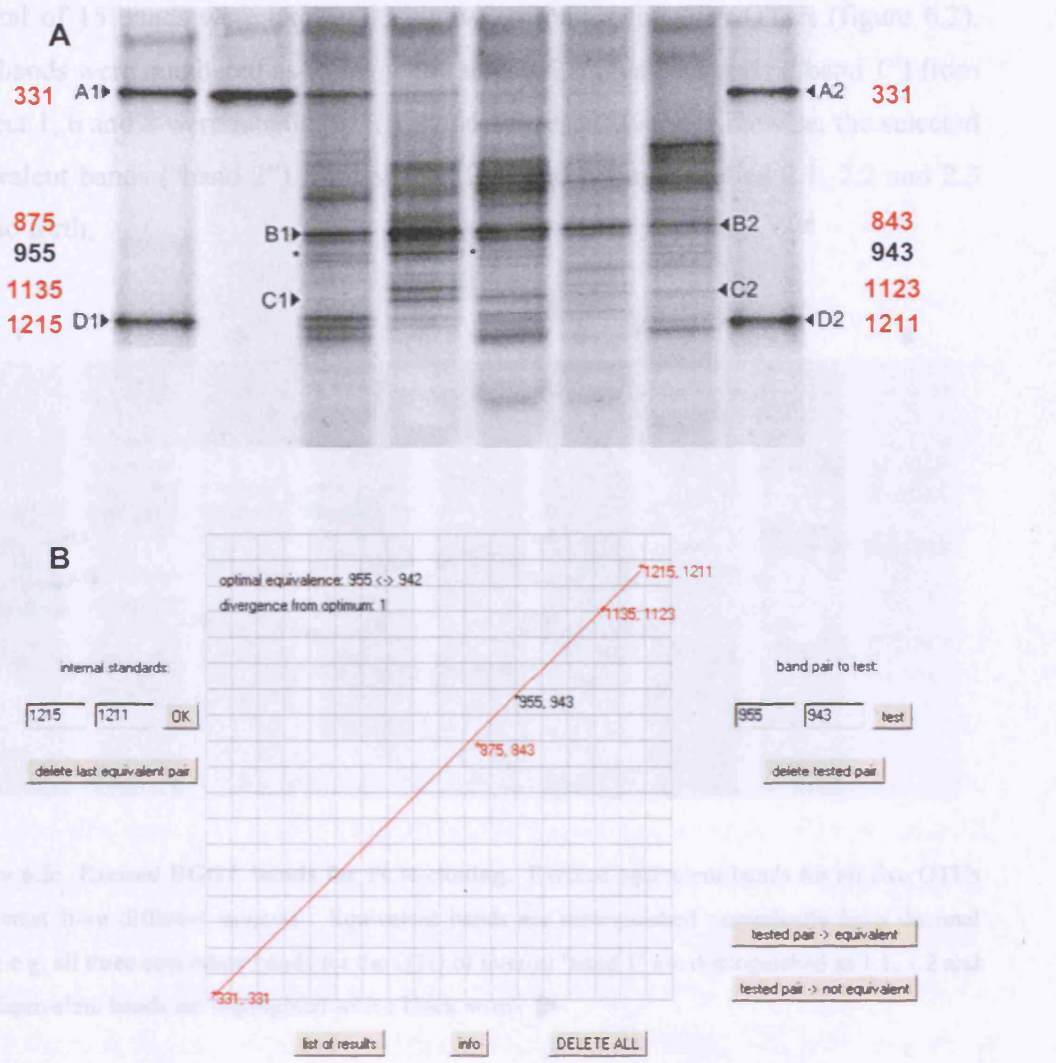


Figure 6.1: (A) Internal standards for EquiBands. DGGE band patterns of 5 different plaque samples. Bands A1/A2 to D1/D2 were treated as internal standards (migration distances, in pixels, have been highlighted in red). The two bands asterisked were checked to determine whether they were equivalent (migration distance in black). **(B) Typical EquiBands result.** Example of graphic output window of the Java applet EquiBands for the internal standards and the 2 bands tested (*). The divergence from optimum was < 3, thus in this case the pair of bands at position 955 and 943 are equivalent

6.3.2 DGGE band excision and PCR-cloning

A total of 15 bands were excised, 3 equivalent bands for all 5 OTUs (figure 6.2). The bands were numbered as follows: the selected equivalent bands ("band 1") from subject 1, 6 and 8 were labelled 1.1, 1.2 and 1.3 respectively. Likewise, the selected equivalent bands ("band 2") from subject 2, 5 and 6 were labelled 2.1, 2.2 and 2.3 and so forth.

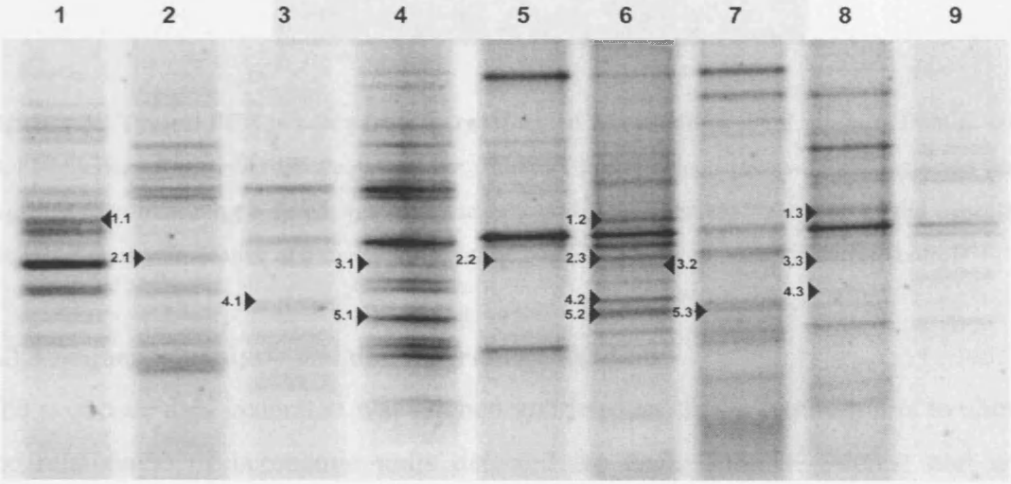


Figure 6.2: Excised DGGE bands for PCR-cloning. Excised equivalent bands for all five OTUs of interest from different subjects. Equivalent bands are distinguished numerically by a decimal value; e.g. all three equivalent bands for the OTU of interest 'band 1' are distinguished as 1.1, 1.2 and 1.3. Equivalent bands are highlighted with a black arrow ▶

The eluted fragments provided a good template for PCR-cloning. Successful ligation of PCR inserts into individual PCR2.1-TOPO vector and the subsequent transformation of competent *E. coli* TOP10 cells occurred most of the time. This was confirmed by PCR, where the expected fragment size should be ca. 370 bp (figure 6.3).

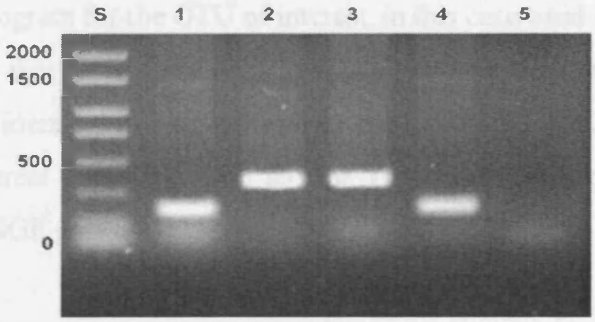
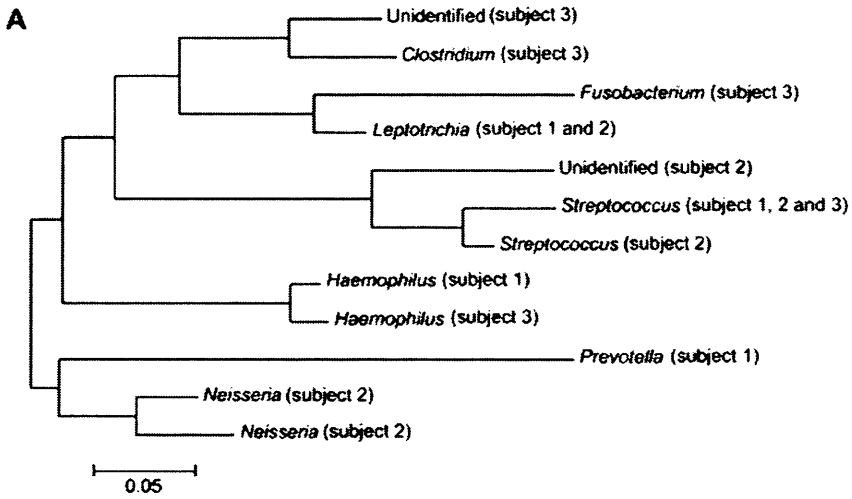


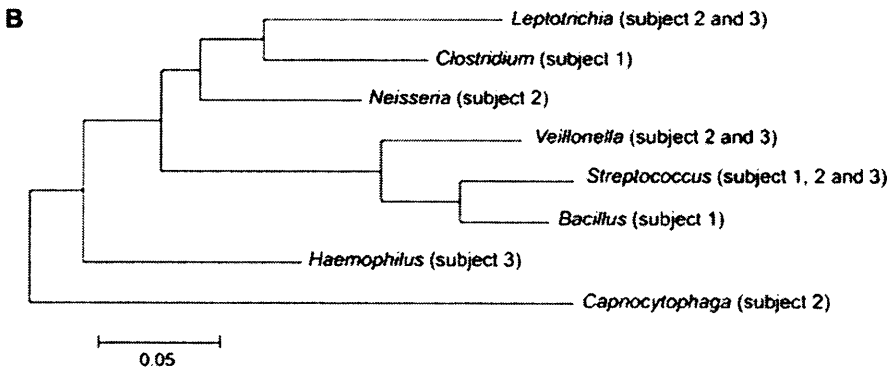
Figure 6.3: Typical PCR product from a transformed *E. coli* clone. PCR products from *E. coli* TOP10 clones. S = molecular weight marker. Lanes 1 and 4 demonstrate smaller fragments than expected thus indicating a failed ligation. Lanes 2 and 3 demonstrate fragments of the expected product size, thus indicative of a successful ligation. Lane 5 consists of water (negative control)

6.3.3 Sequence analysis and dendrogram generation

The sequence data generated was aligned and used to create dendrograms to show the relatedness of taxonomic units detected for each OTU of interest and are presented in figure 6.4 (A-E). The number of taxonomic units shown represents a conservative interpretation of the data because sequences which were slightly different (typically < 5 %) were considered to be the same. Each dendrogram shows an approximate identification of the organism associated with a particular band from which three equivalent bands were cloned and taxa identified. The taxa identified from equivalent DGGE bands from different subjects are demonstrated in the figures 6.4 A-E. Thus, the first equivalent band from subject 1 (figure 6.4 A) contained *Leptotrichia* spp, *Streptococcus* spp, *Haemophilus* spp and *Prevotella* spp, whilst the other two equivalent bands (from subjects 2 and 3) also contained *Clostridium* spp, *Fusobacterium* spp, *Neisseria* spp, as well as unidentified species. Owing to the small size of the usable insert DNA fragment (ca. 162 bp of the ca. 370 bp total fragment), only approximate identification of the species present within each DGGE band could be ascertained. Accurate identification of the genus present was however possible. It is clear from the dendrograms that a wide diversity of unrelated genera were detected at each band position and even from the same physical band.

The distance matrices showed that sequences obtained from the same band could differ from each other by as much as 40%. Figure 6.5 shows the alignment used to generate the dendrogram for the OTU of interest, in this case band 4, before removal of gaps. It shows that certain positions are more variable than others and that the sequences are not identical in length and vary from 138 bp to 162 bp. Thus DNA fragments of different sizes, but with similar Tm appear to migrate to the same position within DGGE gels.





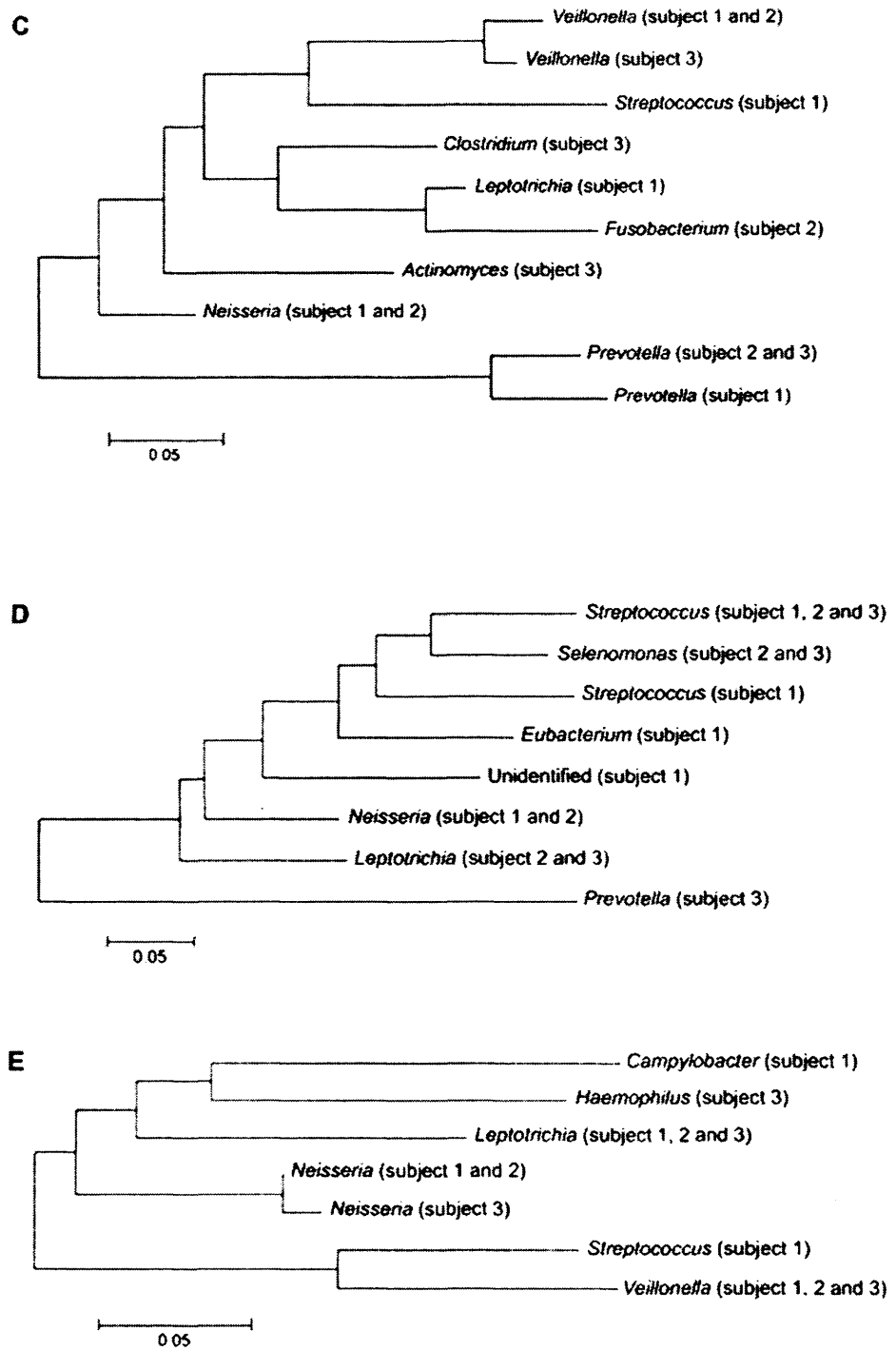


Figure 6.4: Neighbour joining trees showing the phylogenetic relationships between sequences obtained from **(A)** equivalent band 1, **(B)** equivalent band 2, **(C)** equivalent band 3, **(D)** equivalent band 4 and **(E)** equivalent band 5, from DGGE profiles generated from several children. The numbers in parenthesis demonstrate the equivalent bands in which the different genera were detected

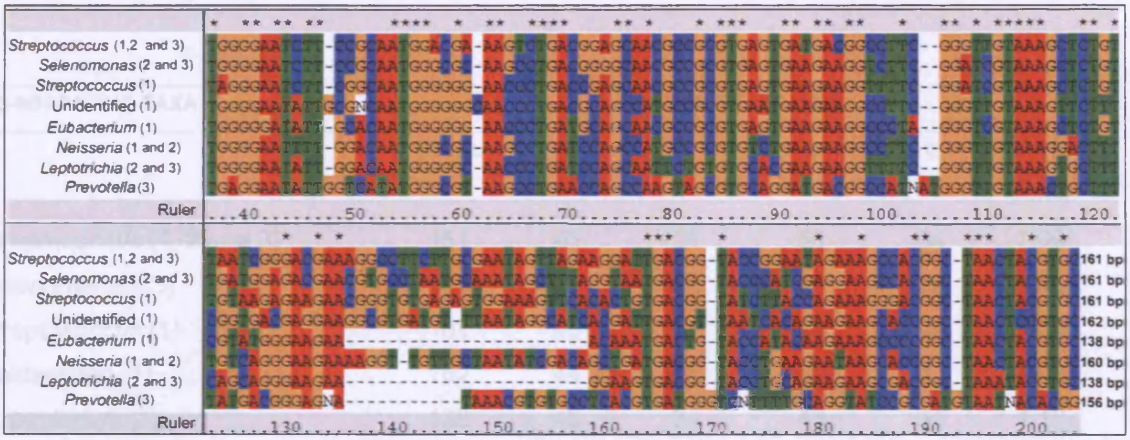


Figure 6.5: Multiple alignments for the sequences of three equivalent DGGE bands. Multiple alignments generated by ClustalX, of the sequences obtained from all equivalent bands of the OTU of interest, band 4. Sequences are identified to genus level and the subjects from which the equivalent sequence was obtained are shown in parentheses

The multiple alignment data demonstrated that each co-migrating taxon contained a different sequence, with different proportions of all four bases (table 6.1). Despite these differences, the PCR products for all eight co-migrating taxa possessed similar Tm (figure 6.6).

CO-MIGRATING TAXA	NUMBER OF BASES					
	TOTAL	A	T	G	C	RATIO GC/AT
Streptococcus (1, 2 and 3)	161	43	34	50	34	1.09
Selenomonas (3)	161	42	32	51	36	1.18
Streptococcus (1)	161	45	32	54	30	1.09
Unidentified (1)	162	44	35	50	33	1.08
Eubacterium (1)	138	40	24	43	31	1.16
Neisseria (1 and 2)	160	43	37	49	31	1.00
Leptotrichia (2 and 3)	138	40	29	45	24	1.00
Prevotella (3)	156	38	39	47	28	0.97
AVERAGE	155	41.6	32.8	48.6	30.9	1.07

Table 6.1: Base distribution in co-migrating taxa. Total number of bases (A, T, G and C) observed for all the co-migrating taxa (figure 6.5) for the OTU of interest, band 4. The subjects from which the equivalent band were obtained are shown in parentheses

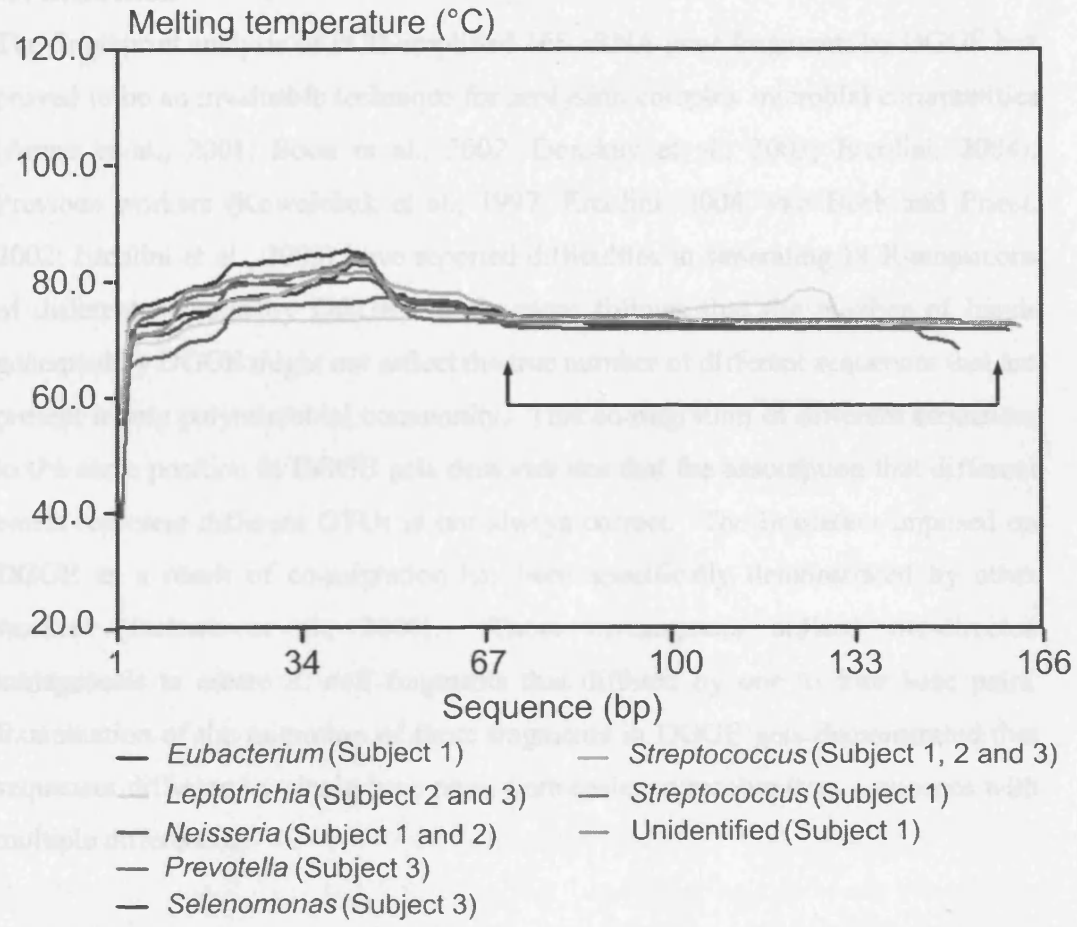


Figure 6.6: WinMelt analysis of seven co-migrating taxa. Different Tm for the PCR fragments of all 7 co-migrating taxa identified from equivalent bands of the OTU of interest, band 4. The subjects from which the equivalent bands were obtained are shown in parentheses. All sequences have a Tm of 70-75°C, except for *Leptotrichia* (75-78°C). The region marked by arrows represents how these sequences would separate by DGGE. Most of the sequences have very similar Tm and thus would be expected to migrate to the same position in a DGGE gel

6.4 Discussion

The fingerprint analysis of PCR-amplified 16S rRNA gene fragments by DGGE has proved to be an invaluable technique for analysing complex microbial communities (Ampe et al., 2001; Boon et al., 2002; Donskey et al., 2003; Ercolini, 2004). Previous workers (Kowalchuk et al., 1997; Ercolini, 2004; van Beek and Priest, 2002; Ercolini et al., 2003) have reported difficulties in separating PCR-amplicons of different bacteria by DGGE. It therefore follows that the number of bands generated by DGGE might not reflect the true number of different sequences that are present in any polymicrobial community. This co-migration of different sequences to the same position in DGGE gels demonstrates that the assumption that different bands represent different OTUs is not always correct. The limitation imposed on DGGE as a result of co-migration has been specifically demonstrated by other workers (Jackson et al., 2000). These investigators utilised site-directed mutagenesis to create *E. coli* fragments that differed by one to four base pairs. Examination of the migration of these fragments in DGGE gels demonstrated that sequences differing by single base pairs were easier to resolve than sequences with multiple differences.

Logistic regression analysis of the DGGE profiles obtained from the dental plaque sampled from children with and without gingivitis (chapter 5) identified differences in the banding patterns between the two cohorts. Logistic regression analysis also demonstrated five specific OTUs that were significantly associated with the presence and absence of gingivitis as well as the presence of the two periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*. Three equivalent bands from different subjects were chosen for each of the five OTUs in order to determine whether equivalent DNA fragments consisted of the same nucleotide sequence. Prior to band excision and sequencing, bands pertaining to different subjects that migrated to the same position were confirmed as being equivalent or not by using the EquiBands applet. Owing to non-uniform migration distance, some bands within a DGGE gel might become spatially distorted at the edges of the gel, thus leading to patterns that are not horizontally aligned (Huber and Peduzzi, 2004). The EquiBands applet

allowed for an improved method of band classification in that it uses simple regression analysis to compare the band profiles of gels with linear gradients. This online tool confirmed that all proposed equivalent bands, for each of the five OTUs, were indeed equivalent. Thus, if DGGE was not limited by co-migration, each of these equivalent bands should contain identical DNA sequences for a specific taxon.

The elution of the excised bands in molecular grade water allowed for the DNA to diffuse out of the polyacrylamide gel. Direct PCR-sequencing of these templates revealed a sequence rich with Ns and a chromatogram that would suggest that the template was mixed. Therefore the eluted DNA from the excised DGGE fragments was cloned in PCR2.1-TOPO vector using the TOPO TA cloning kit. Five random clones per subject, for all three bands of interest, were PCR-sequenced. Phylogenetic analysis of the sequence data obtained from specific equivalent DGGE bands (figure 6.4 A-E) demonstrated that individual DGGE bands were comprised of multiple co-migrating taxa. Additionally, in some cases, the taxa comprising the bands were also shown to be different for "equivalent" bands from different subjects. Thus this study demonstrates that individual DGGE bands may be comprised of more than one OTU and that equivalent bands do not necessarily contain DNA sequences pertaining to the same bacterial taxa. For this reason alone, when working with polymicrobial communities it might be inaccurate to assume that a DGGE band is representative of a single OTU. Perhaps another term of choice for individual DGGE bands from complex communities would be 'co-migrating taxonomic units' (CTU). In addition, multiple alignments of the sequence data (figure 6.5) have also demonstrated that individual co-migrating sequences were rather different and sometimes of varying lengths which in theory may influence migration distance. These fragments migrated to the same position in DGGE gels despite their size difference and a combination of size and GC ratio may have influenced this. In an effort to resolve this, the GC ratio was determined and compared to the length of the product (table 6.1). It was expected that the GC ratio in the sequences with deletions would compensate for the difference in size compared to the longer sequence, or *vice versa*. Nevertheless, when the Tm is

calculated for all the sequences using the WinMelt analysis they were shown to be equivalent (figure 6.6). It therefore seems likely that it is Tm and not fragment size which probably determines how far a PCR product migrates down a DGGE gel.

From these results we can conclude that co-migration does not necessarily imply sequence identity. Interestingly, this analysis also demonstrated that the co-migrating sequences for all three bands of interest were comprised of both Gram-positive and Gram-negative organisms of both coccoid and bacillus morphologies (figures 6.4 A-E). Furthermore, all organisms that were successfully sequenced and identified proved to be from genera which contained oral commensals.

The use of DGGE is of great value in microbial ecology, although it should be recognised that there are limitations, especially when it is applied to the analysis of environmental samples such as dental plaque. Studies of the microbiota of the oral cavity have demonstrated as many as 600 different species (Paster et al., 2001; Kazor et al., 2003) with probably 30-100 taxa per site (Haffajee and Socransky, 1994). The community structure analysis of dental plaque from this study has not detected more than 25 bands in any one profile. This in itself may provide further evidence that co-migration is a common occurrence in the DGGE of complex microbial communities such as dental plaque. Nevertheless many workers have managed to successfully PCR-sequence individual DGGE bands to accurately identify single species. Perhaps this is possible in samples with low species richness as in the case of stilton cheese (Ercolini et al., 2003) where an average of four DGGE bands were observed per sample. These workers managed to PCR-sequence and identify most of their excised bands. Yet even with an average of four DGGE bands per sample (as opposed to dental plaque that tends to have as many as 25 bands per sample), there was evidence of co-migration of two different taxa. This has been further confirmed by other investigators (Blaiotta et al., 2003) who tried to identify different staphylococci from fermented sausages. These workers demonstrated that even though some staphylococci could be correctly identified by DGGE, others could not due to co-migration. Whilst all five bands sequenced in the

current study were shown to be comprised of co-migrating species it would be inaccurate to assume that all the DGGE bands obtained were comprised of co-migrating taxa.

This limitation of co-migration might not be a problem in the DGGE of environmental samples with low species richness. Furthermore, this limitation of co-migration in the DGGE of polymicrobial communities might not be a problem depending on the type of questions one is trying to answer. For example, the effect which an antimicrobial agent has on a microbial community over a period of time might be analysed successfully by DGGE (figure 6.7). In such an experiment, a decrease in bands and band intensities might be observed from the DGGE results. The remaining DGGE bands could later be PCR-cloned and then sequenced to demonstrate which bacterial taxa were resistant to the antimicrobial under test.

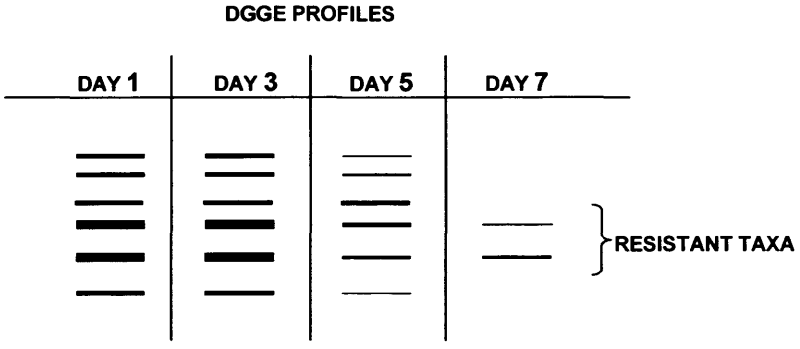


Figure 6.7: Hypothetical effect of antimicrobials on DGGE profiles. Hypothetical DGGE profiles of a microbial community subjected to a fixed concentration of an antimicrobial at regular intervals over a period of seven days. The DGGE result from this type of experiment demonstrates that the antimicrobial had no observable effect on DGGE band numbers or band intensity after three days incubation. After five days incubation, the antimicrobial causes a decrease in DGGE band intensity but there is no change in band numbers. After seven days there is a marked reduction in both the number of bands as well as band intensity

Co-migration does limit the interpretation of these DGGE profiles in that one can not make gross assumptions regarding bacterial diversity and species richness associated with band numbers and band intensities. As an example, it would be

inaccurate to assume that the bacterial diversity and species richness from the microbiota sampled in days 1 and 3 (figure 6.7) are identical. This is elucidated in the hypothetical figure 6.8 where, as an example, the microbiota from dental plaque is characterised by DGGE over a period of 14 days whilst the subject is rinsing with an antimicrobial mouthwash. Like in figure 6.7, one would expect to observe a reduction in band strength and band numbers. Indeed, perhaps new bands may even arise due to the proliferation of resistant organisms that are positively selected for by the mouthwash. After cessation of the 'perturbation' (i.e. once the subject stops using the mouthwash) the DGGE fingerprint might return to its initial pattern. This would suggest that a microbial stability is maintained in the oral cavity post-perturbation. Previous workers (Tannock, 2002) have demonstrated this stability in the gut microbiota of 10 children receiving antibiotic therapy. In seven out of the ten children, the antibiotic treatment resulted in a marked alteration of the bacterial community profile in DGGE gels. The composition of the gut microflora returned to pre-treatment profiles once the therapy was terminated in five out of these seven children.

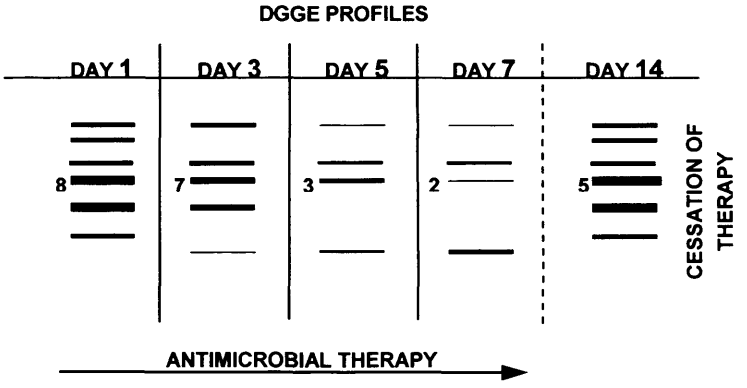


Figure 6.8: Hypothetical effects of mouthwash on dental plaque DGGE profiles. Hypothetical DGGE profiles of dental plaque after seven days rinsing with a mouthwash. Band strength and band numbers become altered throughout the seven day trial. A new band is observed after three days, which may account for taxa that are positively selected for by the mouthwash. Indeed the band intensity for this new band increases over the seven days. A week after the cessation of the trial, the pre-treatment DGGE profile is re-established. The total number of CTUs comprising each band may however change (as exemplified numerically for one band only)

It would be inaccurate to assume that re-establishment of the pre-treatment DGGE profile is synonymous with the re-establishment of the original co-migrating taxonomic richness present within DGGE bands. This is observed in figure 6.8, where five out of the original eight CTUs for a specific band became re-established post perturbation. This means that changes in species richness and bacterial diversity can not be visually interpreted from DGGE profiles. Further work should verify whether the re-establishment of pre-treatment profiles is indeed synonymous with the re-establishment of the pre-treatment taxonomic richness present within individual DGGE bands.

DGGE is therefore useful for comparing differences in the gross microbial composition of different samples. It can be used to demonstrate a change in fingerprint of a sample post-manipulation e.g. analysis of species richness and diversity before and after the use of a mouthwash. Thus DGGE is still a powerful tool if analyses are confined to the study of fingerprints and how these can vary among different environments or how they might change post-perturbation. The limitations of DGGE may be apparent when precise bands identifications are required, especially in species-rich communities such as dental plaque. This might not be the case for species-limited communities where only several taxa may be found to reside. The next and final step of this research will attempt to modify DGGE in order to prevent the limitations imparted on this technique by co-migration.

6.5 Conclusion

In complex microbial communities such as dental plaque, band excision and direct PCR-sequencing might not provide unequivocal identifications as a result of co-migration of different sequences. PCR-cloning of excised DGGE bands and the subsequent PCR-sequencing of individual clones has confirmed that individual DGGE bands from dental plaque are indeed composed of multiple DNA sequences from different organisms.

Chapter 7:
Denaturing gradient gel electrophoresis – gel expansion (DGGEGE)

7.1 Introduction

DGGE of complex polymicrobial communities generates a banding profile, or fingerprint, which has been used to verify changes in the population structure of specific sample groups. This fingerprinting technique has been successfully used to demonstrate the stability of the microbiota of different environments such as human faeces and sludge as examples (Zoetendal et al., 1998; Boon et al., 2000; Vanhoutte et al., 2004b). In these types of investigation the actual fingerprint is important and it is the change or stability in the DGGE profile that is of interest. Other investigators are more interested in determining the specific identity of the OTUs resolved by DGGE. Bands of interest have been previously excised and directly PCR-amplified and sequenced (Ovreas et al., 1997; Ampe et al., 1999; Ercolini et al., 2003) or PCR-cloned and sequenced to successfully identify the taxonomic units of interest (Zwart et al., 1998; Iwamoto et al., 2000). These investigators were primarily concerned in determining key microbial components implicated in processes such as the fermentation of food groups, as in the case of Ampe et al (2001). Other workers attempted to identify the human impact on the microbial diversity of natural environments, as in the case of Iwamoto *et al* (2000) where the microbial components of biostimulated ground waters were analysed. Recently, several investigators (Kowalchuk et al., 1997; van Beek and Priest, 2002; Ercolini et al., 2003; Ercolini, 2004) have reported that band excision and sequencing might not provide unequivocal identification as a result of co-migration of mixed DNA fragments to the same position within DGGE gels. Indeed, the previous chapter demonstrated that co-migration of different taxa occurs in the DGGE of DNA amplified from the 16S rRNA genes present in dental plaque.

There is no research that tackles the limitations of co-migration in any great length. Furthermore, other than PCR-cloning, no further attempts appear to have been made to resolve this limitation. The aim of this work was to attempt to minimise the limitation of co-migration in DGGE by running excised fragments through a shorter denaturing gradient, a process we have termed "denaturing gradient gel electrophoresis gel expansion" (DGGEGE). DGGEGE could resolve individual

DGGE bands consisting of co-migrating taxonomic units (CTU) into true OTUs. DGGEGE fingerprints may therefore offer a complete representation of the microbiota present in subjects with and without gingivitis.

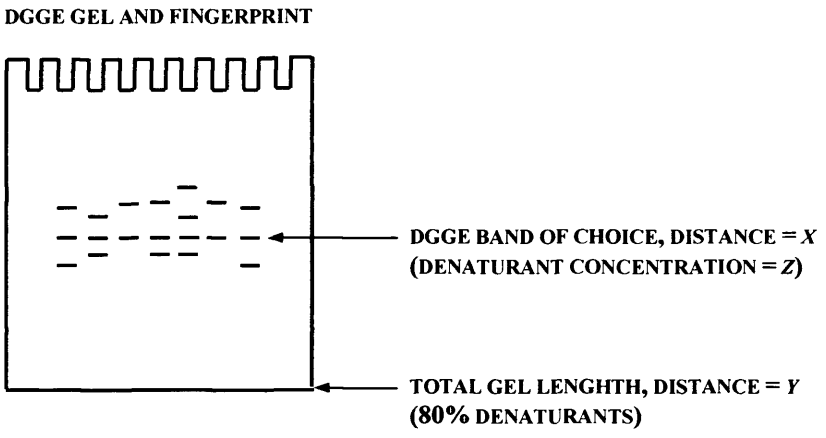
7.2 Materials and methods

7.2.1 Preparation of individual DGGE fragments for DGGEGE analysis

PCR-DGGE (see section 2.8) was carried out using 10 different plaque samples from children with gingivitis (n = 5) and without gingivitis (n = 5). The DGGE profiles were analysed for specific bands that were present in the majority of the subjects profiled. These bands were excised and the DNA fragments were eluted (see section 2.11). The eluted DNA fragments were used as templates in a second touchdown PCR reaction.

7.2.2 Calculating DGGEGE denaturant gradient range

An image of the whole DGGE gel was captured using a UV light transilluminator (AlphaImager) and camera. The distance of electrophoretic migration for the bands of interest within the DGGE gel was measured using the ruler option in the Adobe Photoshop 6.0 software package (San Jose, California). The distance from the loading wells to the bottom of the gel was also measured. The denaturant concentration (Z) at which the band of interest migrated to could then be calculated using both of these distances and the final denaturant concentration of 80% (see figure 7.1). DGGEGE gels were prepared with a lowest denaturant concentration 2% below the value of Z and a highest denaturant concentration 2% above the value of Z.



$$z = \frac{80\% \times x}{y}$$

Figure 7.1: Calculation of denaturant concentration to which DGGE bands migrate. Diagram of DGGE gel illustrating the band, denaturant concentration and calculation of denaturant concentration (z) at which DGGE band of interest migrates to

7.2.3 DGGEGE profiling

Parallel DGGEGE gels containing 10% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) were cast using a DCode system (BioRad laboratories Inc., Hercules, CA, USA). The gels contained a linear gradient of the denaturants urea and formamide, increasing from 50% at the top of the gel to 55% at the bottom (with 100% denaturants corresponding to 7 M urea and 40% [v/v] deionised formamide). PCR products (30 µl) and loading buffer (10 µl) were loaded onto the gels and run at 35 V for 21 hr (735 V hrs) at a constant temperature of 60°C in 7 l of 1x TAE buffer. Gels were stained for 1 hr in 1x TAE containing SYBR Green Nucleic Acid Gel Stain (10^{-4} dilution) (Molecular Probes) and photographed under a UV light transilluminator (AlphaImager). This experiment was repeated to check whether DGGEGE produced reproducible fingerprints.

7.2.4 Cluster analysis of DGGEGE profiles

The DGGEGE profile observed in figure 7.3 A was used in a cluster analysis. The DNA bands that migrated to the same position within the DGGEGE gels were ascribed a number. Band presence in each lane was recorded in binary (band present

= 1 and band absent = 0). This data was clustered using the UPGMA algorithm (Silva and Russo, 2000; Fromin et al., 2002) and a dendrogram was constructed (figure 7.6) using the neighbour-joining method (Saitou and Nei, 1987). Data handling was performed with Paup (Swofford, 2002) and the dendrogram was generated using the Phylip software package (Felsenstein, 1993).

7.2.5 DGGEGE band excision and PCR-sequencing

DGGEGE bands were excised and the DNA fragments eluted. These fragments were amplified by a touchdown PCR (see section 2.8) using the primers 357F and 518R. The PCR products were then directly sequenced. The 3 equivalent DGGEGE fragments were also PCR-cloned and individual clones were PCR-sequenced and identified as detailed in section 6.2.3.

7.3 Results

7.3.1 DGGE profile and band excision

Distinct DGGE profiles were observed for all ten children, the band demarked by the arrow (figure 7.2) was excised from seven out of the ten lanes. The band demarked by the arrow or 'band of choice' stopped migrating at a denaturant concentration of approximately 52-53%.

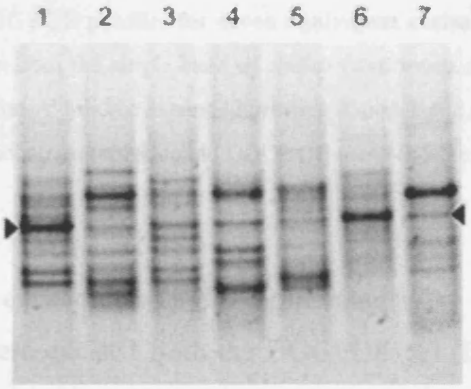


Figure 7.2: DGGE profile from seven subjects demonstrating equivalent bands of interest for DGGEGE. DGGE profiles from dental plaque sampled from seven subjects. The 'band of choice' excised for DGGEGE is demarked by arrows

7.3.2 DGGEGE profiling

PCR-amplified products from the excised and eluted bands (seven in total, see figure 7.2) were subjected to electrophoresis over a very short denaturant gradient (50-55%). The single bands of choice were expanded into mixed profiles (figure 7.3) with an average of 5.4 bands per lane (range of 4-8 DGGEGE bands). This further demonstrated that co-migration of different sequences to the same electrophoretic position does take place in DGGE of dental plaque microbial communities. Separation occurred through a 50.6-51.7% gradient of denaturants. In-duplicate DGGEGE of the same products demonstrated a good degree of reproducibility between runs.

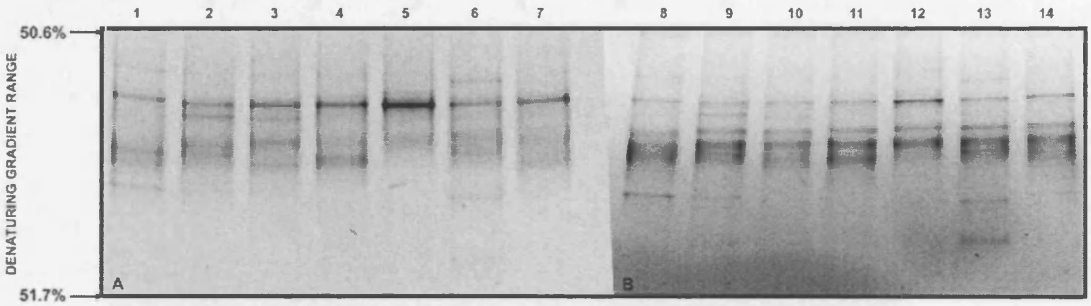


Figure 7.3: Duplicate DGGEGE profiles for seven equivalent excised DGGE bands. Duplicate DGGEGE profiles obtained from the single band of choice from seven different subjects (see figure 7.2). Gel A and B demonstrate duplicate expanded profiles. Lanes 1 & 8; 2 & 9; 3 & 10; 4 & 11; 5 & 12; 6 & 13 and 7 & 14 demonstrate reproducible DGGEGE profiles from the bands of choice excised for the original DGGE gel

7.3.3 DGGEGE band excision and PCR-sequencing

A total of 38 bands were excised from the DGGEGE gel (figure 7.4), direct PCR-sequencing of these bands provided sequence data that showed that the DGGEGE bands were mixed i.e. contained multiple sequences (figure 7.5 A). Three equivalent DGGEGE bands 2, 7 and 12 (figure 7.4) were PCR-cloned and five random clones for each of the three bands were then sequenced. The chromatograms obtained for the different clones demonstrated the presence of single template sequence data (figure 7.5 B). This would suggest that co-migration of different sequences also occurs in DGGEGE. PCR- sequencing of the clones obtained from the equivalent bands 2, 7 and 12 (figure 7.4) demonstrated that these bands were indeed mixed with several co-migrating taxa (table 7.1). *F. nucleatum* and various streptococci were identified from all three bands along with two or three other taxa. Gram-positive and Gram-negative taxa were present in all three bands.

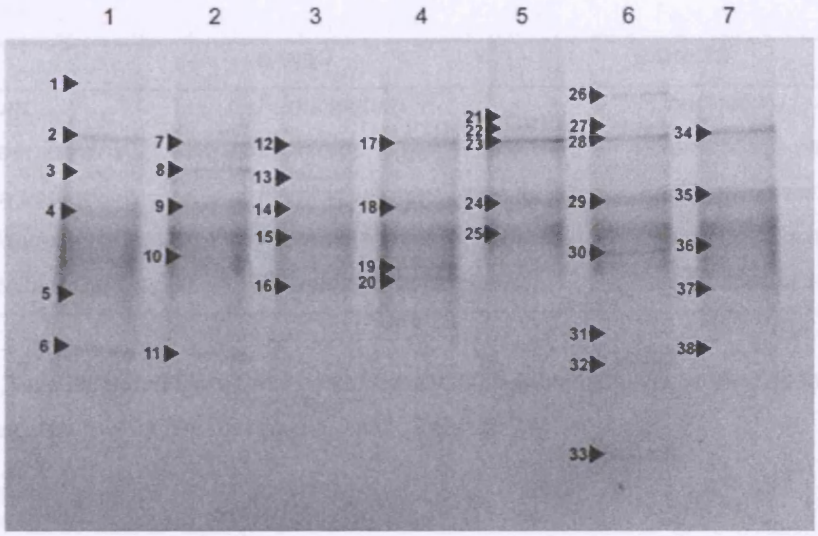


Figure 7.4: DGGEGE bands excised for sequencing: 38 bands were excised in total

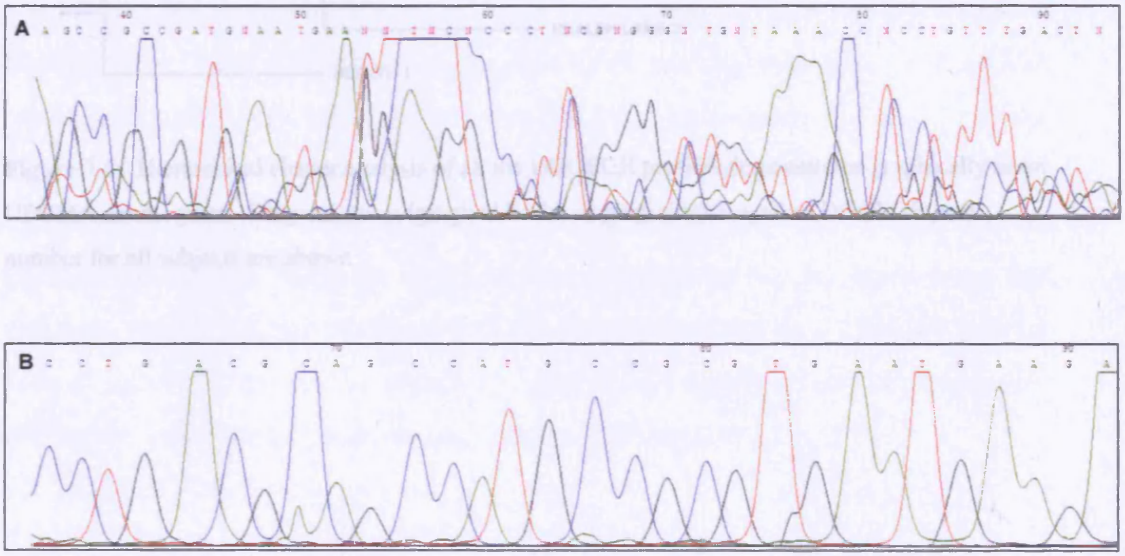


Figure 7.5: Partial chromatograms demonstrating nucleotide sequence data generated from (A) a DGGEGE fragment that has been directly PCR-sequenced and **(B)** single clone from the same DGGEGE fragment

BAND 2	BAND 7	BAND 12
F. nucleatum	*F. nucleatum*	*F. nucleatum*
Gemella haemolysins	*P. micros*	*Haemophilus parahaemophilus*
Leptotrichia spp.	*Porphyromonas catoniae*	*Porphyromonas catoniae*
Neisseria flavescens	*Prevotella shahii*	*Streptococcus mitis*
Streptococcus cristatus	*Streptococcus cristatus*	*Streptococcus sanguinis*

Table 7.1: Taxa identified from three equivalent DGGEGE bands . Taxa identified from the three equivalent excised DGGEGE bands 2, 7 and 12 (figure 7.4)

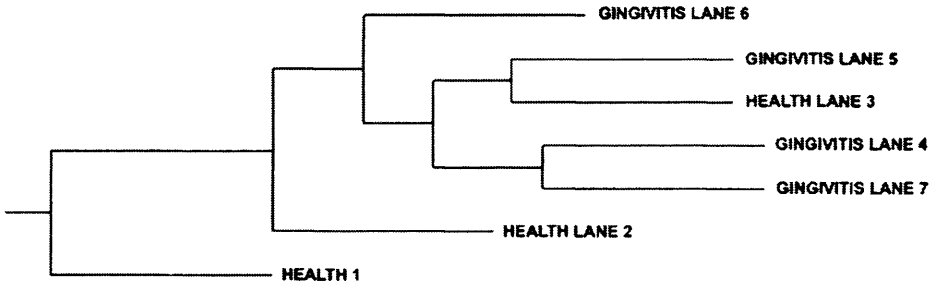


Figure 7.6: Hierarchical cluster analysis of all the DGGEGE profiles demonstrated graphically as an UPGMA dendrogram. Gingival status (gingival health or gingivitis) as well as DGGEGE lane number for all subjects are shown

7.4 Discussion

The purpose of this work was to develop a technique that would overcome the limitations imposed on DGGE by co-migration. The basic principles of DGGEGE are based on increasing the resolution of the technique sufficiently to separate templates which co-migrate when "normal" (40%-80%) DGGE is used. It would be possible, although time consuming, to excise every DGGE band from a single lane and then attempt to resolve each individual DGGE band into its constituent taxonomic units by DGGEGE. By aligning each DGGEGE profile accordingly, we hoped to be in a position to generate a composite fingerprint that truly represented the dental plaque microbiota (figure 7.7).

DGGEGE of single DGGE bands into multiple band profiles provides further evidence that the co-migration of different sequences occurs in the DGGE of DNA fragments amplified from the 16S rRNA genes present in dental plaque. By using plaque sampled from children with and without gingivitis, it could be discerned whether there were similarities in the DGGEGE profile from equivalent DGGE bands from children in both cohorts. An UPGMA dendrogram (figure 7.6) of the DGGEGE profiles from an equivalent band (figure 7.3) demonstrated a level of distinction between the banding patterns of both cohorts. Due to the small number of equivalent bands tested (n = 7), no real conclusions can be made from this analysis. Indeed a larger sample size might demonstrate that the DGGEGE profiles of both cohorts might cluster separately, thus demonstrating significant taxonomic differences between the gingivitis and no gingivitis subjects.

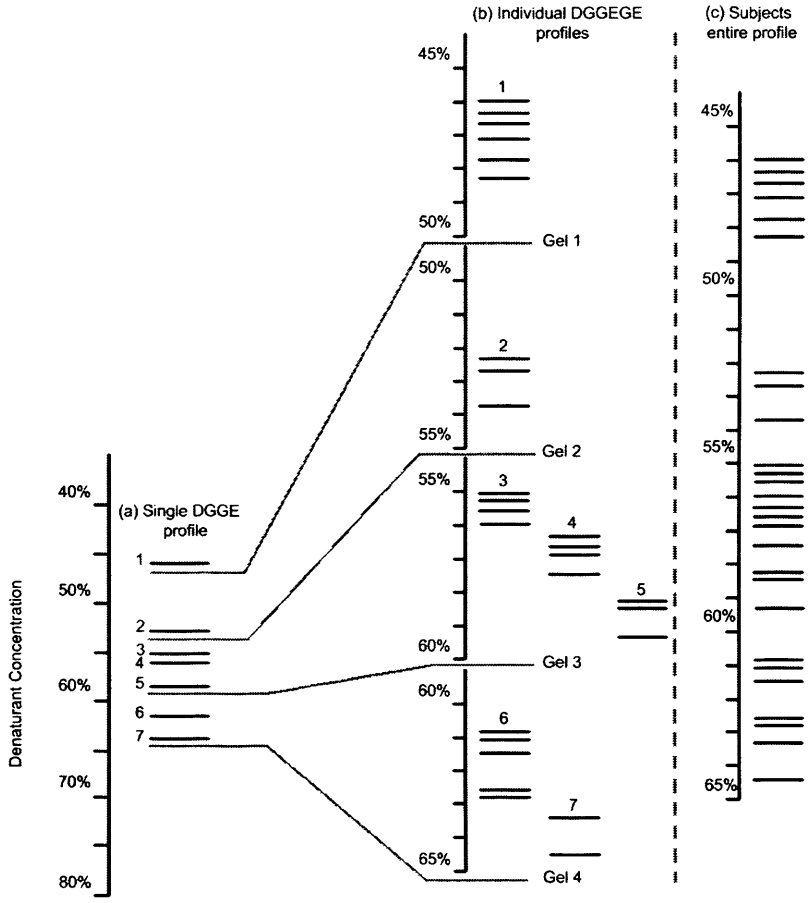


Figure 7.7: Schematic summarising the principles underlying DGGEGE. (A) Individual DGGE bands are first excised and the eluted DNA is amplified by touchdown PCR. The denaturant concentrations to which individual bands migrate to can be calculated in order to discern the shorter concentration gradients required for resolving out CTUs into individual OTUs. (B) Four DGGEGE gels with short denaturant gradients (45-50%, 50-55%, 55-60% and 60-65%) could then used to resolve the excised DGGE bands into multiple other taxonomic units. Some excised DGGE bands, such as bands 3-5 are resolved on the same DGGEGE gels. (C) Composite DGGEGE profile of the excised bands from a single DGGE lane

A total of 38 DGGEGE bands were excised (figure 7.4) and their DNA eluted and PCR-sequenced. The initial sequence data obtained from direct PCR-sequencing of the excised DGGEGE bands demonstrated that these were comprised of mixed multiple sequences (figure 7.5 A). Three equivalent bands (2, 7 and 12, figure 7.4) were excised, cloned and sequenced. The chromatograms obtained from each individual clone demonstrated that they were not mixed (figure 7.5 B). It may be assumed that the other 35 DGGEGE bands will probably also contain multiple co-migrating sequences. This demonstrates that, as in DGGE, DGGEGE bands appeared to be comprised of mixed co-migrating sequences belonging to different species of oral bacteria. Thus DGGEGE could not resolve CTUs from equivalent DGGE profiles into individual OTUs. Perhaps when assessing extremely complex whole microbial communities, such as those that exist in the oral cavity, a DGGE approach is inappropriate and the use of a PCR-cloning approach is called for.

7.5 Conclusion

DGGE analysis of the community structure of dental plaque is limited by the co-migration of different sequences to the same migratory position within the gels.
These co-migrating sequences can be separated by electrophoresing DGGE bands through a smaller denaturant gradient (DGGEGE). DGGEGE of equivalent DGGE bands does not generate the same banding profile. This not only further substantiates the fact that co-migration is occurring, but also demonstrates that co-migration does not imply sequence identity. As a result of this co-migration of DNA from multiple taxa, perhaps individual DGGE bands should not be referred to as operational taxonomic units (OTUs) but rather co-migrating taxonomic units (CTUs). Cloning and subsequent sequence analysis of DGGEGE bands have demonstrated that these bands also comprise mixed co-migrating sequences, thus DGGEGE can not resolve CTUs into true OTUs. In the light of such evidence, DGGE as a fingerprint technique for the analysis of complex microbial communities might best be suited for observing relative changes in the banding profiles pre- and post perturbation. For a more complex phylogenetic analysis of such complex communities, PCR-cloning appears to remain the best method of choice.

Chapter 8:
Final conclusions and scope for further work

8.0 Final conclusions and scope for further work

Epidemiological studies have demonstrated that, unlike periodontitis, gingivitis is common both in children and adults (Papapanou, 1996; Jenkins and Papapanou, 2001). Gingivitis may be influenced by a number of factors ranging from hormonal fluctuations to social habits such as smoking. These confounding factors were eliminated by recruiting fit, healthy pre-pubertal children who had not taken any antibiotics during the three months prior to plaque sampling. The presence or absence of gingivitis around the gingival margin of the lower left or right first permanent molar teeth was mostly likely due to poor oral hygiene and dental plaque build-up. Analysis of the dental plaque samples was primarily conducted by observing for differences in the prevalence of the periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in both cohorts. This was followed by an attempt to analyse both the functional and structural diversity of the bacterial communities present in both children with and without gingivitis.

Dental plaque was sampled from the lower first permanent molars because these are the first permanent teeth to erupt and periodontal disease does not usually affect the primary teeth in healthy children. Molars are also the teeth that are most frequently lost as a result of periodontal disease (Hirschfeld and Wasserman, 1978; Matthews et al., 2001). This is because the anatomy of molars favour the retention of bacterial deposits and makes periodontal debridement, as well as oral hygiene procedures, difficult (Matthews and Tabesh, 2004). Furthermore, local aggressive periodontitis which principally affects young patients (Albandar and Tinoco, 2002) tends to affect only the incisors and molars (Beck et al., 1992; Ebersole et al., 1994).

Several periodontal pathogens have been highlighted as key microbial aetiologies for the inception and progression of periodontal diseases (Darby and Curtis, 2001). Among these, *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* have been implicated as the main microbial aetiological agents of periodontal disease (Tanner et al., 1979; Slots et al., 1986; Wojicicki et al., 1986; Bragd et al., 1987; Dzink et al., 1988; Haffajee and Socransky, 1994; Haffajee et al., 1999; Ximenez-

Fyvie et al., 2000b; Socransky and Haffajee, 2002). The presence of these pathogens has been reported in the oral cavity of very young children (Tanner et al., 2002b). This present research further demonstrates that these organisms are indeed present in pre-pubertal dental plaque and hence supports the claim that they may be truly endogenous in the oral cavity. However, not all children sampled in the multiplex PCR study were positive for all three organisms. Indeed some children appeared not to harbour any of the three pathogens at all. This does not exclude the possibility that the pathogens were indeed present, but below the threshold of detection of the multiplex PCR assay. Furthermore, because dental plaque was only sampled from either the lower left or lower right first permanent molar teeth, it would be reasonable to assume that these pathogens might have been present at other sites. It is also unclear if the early colonisation of these three pathogens in children can be regarded as a risk factor for future development of periodontal disease.

It was anticipated that the three pathogens would have been detected more frequently in subjects with gingivitis, yet this was not the case. There was no significant difference between the detection frequencies of *P. gingivalis* and *A. actinomycetemcomitans* between the two cohorts. There was a significantly greater detection of *T. forsythensis* in children without gingivitis. This suggests that other factors such as the actual numbers of individual pathogens might be responsible for causing gingival inflammation, which further substantiates the principles of the ecological plaque hypothesis. The hypothesis proposes that organisms associated with disease can be present at healthy sites but at levels too low to be clinically significant (Marsh, 1994). The prevalence data from the multiplex PCR study can only verify whether the three pathogens were present or absent, but cannot measure the actual numbers of the pathogens present at the sample sites. There might indeed be significant differences between the two cohorts, but only with regard to pathogen numbers and not just their prevalence. Future work on these plaque samples should include quantitative real-time PCR for the three periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* (Morillo et al., 2004).

The significantly higher detection rate of *T. forsythensis* in children without gingivitis was unexpected. This higher prevalence in children without gingivitis can be explained in terms of possible cross-reactivuty of the *T. forsythensis*-specific PCR primer, BfF, for other phylogenetically related organisms such as the novel *Bacteroides* phylotype, oral clone BU063. Future work might involve the analysis of *T. forsythensis* and the oral clone BU063 in the dental plaque of children with and without gingivitis. This might be achieved by PCR-sequencing less homologous target regions, such as the intergenic spacer regions (ISR) between the 16S and 23S rRNA genes of both organisms.

The multiplex PCR study did not differentiate between different clonal types of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. It is possible that specific virulent clones of these three pathogens colonise subjects with periodontal disease whilst less pathogenic clonal types colonise subjects with no periodontal disease. Genotypic characterisation of *P. gingivalis* isolates from subjects with periodontitis has revealed an extensive heterogeneity and as many as 100 different clonal types of this pathogen (Amano et al., 2004). The same groups of workers have further reported an apparent close association between periodontitis and *P. gingivalis* clones possessing specific genotypes (Amano et al., 1999; Nakagawa et al., 2000). The *P. gingivalis* fimA gene encoding fimbrillin, a subunit of fimbriae, has been classified into 5 genotypes (types I to V) based on their nucleotide sequences (Dickinson et al., 1988). In healthy subjects, clonal types of *P. gingivalis* with the fimA type I and V genotypes were more prevalent (Amano et al., 2000). In contrast, most of the periodontitis patients carried type II and IV fimA organisms (Amano et al., 2000). Thus there appears to be different clonal types of *P. gingivalis* associated with both health and disease.

A large number of different clones of *A. actinomycetemcomitans* in patients with and without periodontal disease are evident (Poulsen et al., 1994; Kaplan et al., 2002). Many of these different clonal types have been isolated from subjects with periodontitis and hence they may all have a pathogenic potential (Kaplan et al.,

2002). Some clonal types of *A. actinomycetemcomitans*, such as the JP2 strain, are highly virulent in that they are reported to express higher levels of leukotoxin than other, less virulent clonal types (Brogan et al., 1994). These JP2-like strains appear to be more prevalent in individuals from African descent as opposed to Asians and Caucasians (Haubek et al., 1995; Contreras et al., 2000).

All of these genotypic distribution studies have reported multiple clonal types of the three periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Prevalence data and indeed quantification of the actual numbers of these three pathogens by real-time quantitative PCR will not provide any insight into the different clonal types that may be present in pre-pubertal children with and without gingivitis. It may be that subjects with gingivitis harbour more virulent clonal types of the three pathogens than children without gingivitis. Future work might include a genotype distribution analysis of these three periodontal pathogens isolated from subjects with and without gingivitis by using techniques such as multilocus enzyme electrophoresis (MEE) and/or restriction fragment length polymorphism (RFLP) (Haubek et al., 1995). Recent workers have also used DGGE to distinguish between different serotypes of *A. actinomycetemcomitans* in *in vitro* studies (Ihalin and Asikainen, 2006). Future work may therefore also include the development of PCR-DGGE protocols that identify the different clonal types of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* in subjects with and without gingivitis.

The development of periodontal disease is a complex process involving dental plaque as the main aetiological factor (Löe and Holm, 1965; Socransky, 1970; Lindhe et al., 1973; Bosman and Powell, 1977; Page, 1986;Breuer and Cosgrove, 1989; Mariotti, 1999). Dental plaque as a whole could therefore be considered as a 'pathogenic entity' responsible for causing disease. Yet severe periodontal destruction is only prevalent in a small subset of subjects. Most researchers have tried to determine whether diseased subjects differ from non-diseased subjects in (i) the prevalence, numbers and clonal types of specific pathogens, and (ii) varying host

responses to specific pathogens. The more holistic approach of analysing dental plaque as a whole, in order to characterise health and disease, appears to have lagged behind. Community analysis via substrate utilisation profiling with Biolog plates has been used to analyse bacterial communities of several environments (Konopka et al., 1998). The data from these experiments have been used in two ways: (i) to quantify differences among specific environmental samples, and (ii) to assess the functional diversity of microbes in ecosystems (Konopka et al., 1998). The functional diversity of a microbial community has been defined as the numbers, types, activities and rates at which a suite of substrates is utilised (Zak et al., 1994). The 95 different substrates in Biolog GN plates, classified by Garland and Mills (1991), provides a wide array of carbon substrates that should reflect, both qualitatively and quantitatively, the diversity of substrates available in most natural environments. It is logical to assume that dental plaque communities will generate a substrate utilisation profile thus giving an insight into its functional diversity. An analysis of the substrate utilising profile of subjects with and without gingivitis may indeed provide an insight into which carbon substrates are predominantly utilised in both subject groups. By comparing the prevalence data for *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis* with substrate utilisation profiles, perhaps a substrate fingerprint could have been determined to not only predict between the two cohorts but also to predict the presence of these three periodontal pathogens.

Community level physiological profiling (CLPP) by measuring substrate utilisation profiles or the 'metabolome' of dental plaque samples was not attempted. This was because preliminary work with single streptococcal isolates did not provide reproducible Biolog profiles. Another fundamental problem with the use of carbon substrate utilisation patterns to directly assess microbial diversity is that Biolog profiles are insensitive to changes in population structure. This is a consequence of the 'metabolic redundancy' in communities, i.e., many species have the genetic potential to use a specific substrate (Konopka et al., 1998). Thus, large changes in species genetic diversity may yield a very small change in the functional diversity as

assayed by Biolog plates. This is because in a diverse microbiota it is very likely that there is a substantial metabolic redundancy for all these 95 carbon substrates, thus the loss of species that degrade one of these substrates will not result in an altered Biolog profile.

It was of particular interest to determine which carbon substrates were preferentially utilised by the plaque microbiota of subjects without gingivitis. By promoting the utilisation of such substrates it might be possible to confer a selective bias over the microbial taxa that are present in gingival health. Further work should be conducted to investigate potentially 'prebiotic' substrates to promote gingival health. The term Prebiotic been defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in an environment," (Schrezenmeir and de Vrese, 2001). It might be possible to prevent the microbial shift from health to gingivitis by providing subjects with a range of prebiotic substrates that select positively for organisms associated with gingival health.

The culture-dependent fingerprint technique CLPP was abandoned due to issues concerning reproducibility of results and thus a molecular approach, DGGE, was used in an attempt to answer the following questions:

(i) Is there a specific microbiota that influences the prevalence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis?*

(ii) Is there a specific microbiota that distinguishes between subjects with and without gingivitis?

DGGE generated DNA band profiles that appeared to be distinct among patients, within and between both cohorts. Logistic regression analysis demonstrated that the DNA banding profiles from the subjects with and without gingivitis were substantially different in that the presence or absence of gingivitis was correctly classified in 84% of the subjects. Furthermore, this type of analysis of the DGGE

fingerprints also confirmed that individual bands were significantly associated with the presence of *P. gingivalis* and *A. actinomycetemcomitans*. No bands appeared to be associated with the presence or absence of *T. forsythensis*. Thus at first glance DGGE appears to answer both ofthe above questions.

Primarily, there appears to be a banding pattern that can distinguish the DGGE profiles between both cohorts. This suggests that sufficient differences might exist between the microbiota of both cohorts. Logistic regression analysis pinpoints specific DGGE bands that are associated with a particular outcome. In this study the outcomes were, (i) whether the subject had gingivitis or not and (ii) the prevalence of the three individual periodontal pathogens *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythensis*. Logistic regression analysis demonstrated five bands that were associated with these outcomes. Several attempts to verify the sequence identity of these DGGE bands demonstrated that they were mixed. This can be explained in terms of the co-migration of several DNA fragments from different taxa to the same electrophoretic position. These co-migrating DGGE bands consisted of heterologous DNA sequences with similar T-m. Thus when analysing the DGGE profiles obtained from complex polymicrobial communities, it may be inaccurate to assume that any given band is representative of a single OTU. Perhaps in such instances individual DGGE bands should be referred to as co-migrating taxonomic units (CTU).

Personal communications with other workers has revealed that co-migration does limit the DGGE analysis of polymicrobial communities amplified with universal PCR primers. More importantly, these personal communications have also revealed that co-migration does not appear to present as much of a problem when using genus-specific primers in DGGE with shorter denaturing gradients. Future work might involve the DGGE characterisation of different genera present in dental plaque samples of subjects with and without periodontal disease. Indeed DGGE might also be used with species-specific primers in order to identify the presence of

different clonal types of a periodontal pathogen in subjects with and without periodontal disease.

Another limitation of DGGE is the separation of only relatively small fragments, up to 500 bp in size (Muyzer and Smalla, 1998). This limits the amount of sequence information that might be used for phylogenetic analysis as well as probe design. A further problem in the study of community diversity on the basis of 16S rRNA genes using DGGE is the presence in some bacteria of multiple copies of the rRNA operons with sequence microheterogeneity. DGGE can visualise these differences and hence might lead to an overestimation of the number of bacteria within a community (Muyzer and Smalla, 1998).

The future use of DGGE in the characterisation of dental plaque should be limited to genera- and species-specific primers. Furthermore, simple DGGE patterns may also be obtained by using PCR primers that target functional genes, which are only present in particular bacterial populations (Muyzer and Smalla, 1998). The statistical methods used in this study, namely (i) the Shannon-Wiener index for measuring bacterial diversity, (ii) hierarchical cluster analysis and (iii) logistic regression analysis, might prove useful in the investigation of any differences in the bacterial community structure between cohorts at a genus level. Clearly, for the analysis of whole microbial communities that span multiple genera, PCR-cloning still remains the primary method of choice.

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List of Appendices

Appendix 1: Composition of Reduced Transport Fluid (RTF)

Stock Solution 1	
Dibasic potassium phosphate	0.6 g
Distilled water	100 ml
Magnesium Sulphae stock solution	
Magnesium sulphate	2.5 g
Distilled water	100 ml
Stock Solution 2	
Potassium chloride	1.2 g
Ammonium sulphate	1.2 g
Monobasic potassium phosphate	0.6 g
Magnesium sulphate stock	1 ml
Distilled water	99 ml
Sodium Carbonate Solution	
Sodium carbonate	0.8 g
Distilled water	10 ml
Preparation for 100 ml	
Stock solution 1	7.5 ml
Stock solution 2	7.5 ml
Sodium carbonate	0.5 ml
Distilled water	80 ml

Autoclave solutions at 121°C for 15 min and allow to cool. Once cool, add a filter-sterilised solution of dithiothreitol (Sigma; 0.02 g in 5 ml distilled water).

Appendix 2: Composition of Electrophoresis Buffer Tris-acetater EDTA (TAE)

Concentrated stock solution (per litre)

For 50x concentrated stock

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Publications and presentations

Scientific papers

1. Gafan, G.P. and D.A. Spratt. 2005. Denaturing gradient gel electrophoresis gel expansion (DGGEGE) – An attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *FEMS Microbiol. Lett.* 253:303-307.

2. Gafan, G.P., V.S. Lucas, G.J. Robers, A. Petrie, M. Wilson and D.A. Spratt. 2005. Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *J. Clin. Microbiol.* 43(8):3971-3978.

3. Gafan, G.P., V.S. Lucas, G.J. Roberts, A. Petrie, M. Wilson and D.A. Spratt. 2004. Prevalence of periodontal pathogens in dental plaque of children. *J. Clin. Microbiol.* 42(9):4141-4146.

Book chapters

1. Gafan. G., V. Lucas, M. Wilson and D. Spratt. (2003). Community analysis of the microflora of dental plaque associated in health and gingivitis via a denaturing gradient gel electrophoresis approach. *In* Biofilm Communities: Order from Chaos? Bioline, Cardiff.

2. Gafan, G.P., D. Elliot, M. Wilson and D. Spratt. (2005). Profiling the oral microbiota associated with the prevalence of three periodontal pathogens. *In* Biofilms: Persistence and Ubiquity. The Biofilm Club, The University of Manchester.

Poster presentations

1. Gafan. G.P., M. Wilson and D.A. Spratt. 2005. Use of short gradient DGGE gels to analyse previously excised individual DGGE bands. ASM meeting on Beneficial Microbes, Lake Tahoe, Nevada.

2. Gafan, G.P., V.S. Lucas, G.J. Roberts, M. Wilson and D.A. Spratt. 2005. Limitations of DGGE as a result of co-migration. ASM meeting on Beneficial Microbes, Lake Tahoe, Nevada.

3. Gafan, G.P., V.S. Lucas, G.J. Roberts, A. Petrie, M. Wilson and D.A. Spratt. 2003. Community analysis of the microbiota of dental plaque associated in health and gingivitis using a denaturing gradient gel electrophoresis. ASM meeting on Biofilms, Victoria, British Columbia.

4. Gafan, G.P., V.S. Lucas, G.J. Roberts, M. Wilson and D.A. Spratt. 2002. Detection of periodontal pathogens from dental plaque of prepubertal children using multiplex PCR. IUMS meeting, Paris, France.